SAPIENZA
Università di Roma
Facoltà di Scienze Matematiche Fisiche e Naturali

DOTTORATO DI RICERCA
BIOLOGIA CELLULARE E DELLO SVILUPPO

XXIV Ciclo
(A.A. 2010/2011)

"ROLE OF NOT4 AND JHD2 IN REGULATION OF HISTONE H3 METHYLATION AND TRANSCRIPTION OF RIBOSOMAL PROTEIN GENES IN YEAST"

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SUMMARY

SUBMITTED PUBLICATION

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“Not4-Jhd2 interplay regulates histone H3 methylation and transcription of ribosomal protein genes in yeast”.

Nucleic Acids Research - Manuscript NAR-00051-2012.

INTRODUCTION

Chromatin

1.1 Chromatin and gene expression

All living beings retain their hereditary information in the form of double-stranded DNA molecules that is the depositary of necessary instructions for cells to live, grow and multiply. The DNA molecule also has a second important function that convert the information contained in it in processes of transcription and translation. In eukaryotic cells DNA is contained within the nucleus, of only a few microns in diameter. This implies that the DNA must be highly compressed in order to enter the restricted space, folds back to form higher order structures.

The DNA is organized into chromosomes, which consist of fibers, called chromatin, which is structurally divided into highly condensed heterochromatin and actively transcribed, and less condensed euchromatin and transcriptionally inactive. Chromatin consists of DNA and proteins that are generally divided into two groups: the histones, basic proteins and small non-histone proteins with structural features, enzymatic and regulatory. The association between histones and DNA is the basic unit of chromatin, the nucleosome, whose function is not just a simple structural,

but is involved as a multiprotein complex, in many aspects of cellular life, from the telomeric silencing to transcription.

Nucleosomes are the basis of a high order packaging structure, chromatin. Compared to naked DNA, nucleosomal DNA is less accessible to regulatory proteins and regulatory processes. The exact positions of nucleosomes therefore influence several cellular processes, including gene expression, chromosome segregation, recomsination, replication and DNA repair).

In vivo experiments with nucleases Micrococcal of chromatin, have allowed to define some parameters of the structure nucleosomale. This is an enzyme capable of cutting the DNA at random when it is not bound to proteins, so, permanently bound proteins to DNA, such as nucleosomes, offer protection to enzymatic digestion. The results define a length of repeated DNA, whose size varies from 180 to 200 bp. Is known that over 80% of DNA in eukaryotic cells is organized in the form of repeated nucleosomes, each of which consists of 200 bp of DNA associated with histone octamer defined core consisting of ($[H2A, H2B, H3, H4]_2$). Treatment with acid solvent of octamer reveals its composition: it consists of only four proteins, H2A, H2B, H3 and H4 are present in duplicate to form a tetramer,$(H3)_2$ $(H4)_2$ (Kornberg

et al, 1974 and Roark et al., 1974) and two H2A-H2B heterodimers; these proteins are histones. Histones are small basic proteins with a molecular weight that is approximately between 11 and 16 kDa.

Histones H3 and H4 are surprisingly well conserved through evolution, suggesting that they should have a key role, while the other histones are less conserved and have been found variants with different properties.

DNA, therefore, is organized around the histone octamer in 1.65 turns, macking a left handed superhelix. The superhelix has a diameter of 41.8 Å and the curve is accentuated in some locations. Histones are associated in such a way that the tetramer lies at the center of the nucleosome and the two dimers are located outside of the tetramer to surround himself. The arrangement thus obtained has a double symmetry. The formation of pairs of histones to form the tetramer and two dimers, is the result of the complementarity of their withdrawal, but is also determined by the interactions that are established between DNA and histones.

When the DNA is wrapped around the nucleosome is distorted in several places and her propeller pitch varies from 10.5 base pairs that in solution, to 10.2 base pairs when bound to octamer.

This altered periodicity generates an array of grooves, major and minor, between the two curves near the DNA and creates channels which we put the histone tails (Luger et al., 1997).

In the formation of the nucleosome, at the molecular level, interactions of DNA with histone proteins are due to various types of bonds:

a) Hydrogen bonds that develop among phosphate groups of DNA and amino residues of histones.

b) Ectrostatic interaction with the basic side chain

c) Interactions with polar groups deoxyribose (Kornberg et al, 1999).

The lack of specific binding with the nucleotide bases has important functional consequences because histones can bind without specific DNA sequence.

In higher eucaryotes there is a further histone, H1, linker histone said, that does not seem important for the formation of the nucleosomal particle but apparently involved in the formation of higher order (Van Holde et al, 1988 and Wolffe et al, 1998).

The observation of chromatin at different ionic conditions in vitro, revealed different structures that reflect higher order chromatin organization (Thoma et al., 1979) (Fig. 1).

The “beads on a string” structure, the also called 10 nm fiber, is the first level of structural organization and determines a degree of compaction of about 7 times.

At higher salt concentrations, this structure further condenses into a fiber of 30nm, interpreted throught the “solenoid model” in which the 10 nm fiber is wound on itself in a left-handed superhelix with six nucleosomes per turn of helix and an increase in the level of compaction of the other 7 times (Thoma et al., 1979).

The next level of chromatin compaction is the metaphase chromosome, which imposes a further compression of 250 times.

Chromatin is a highly dynamic changes continuously with respect to key moments of the cell cycle:

a) Replication (Flanagan et al, 1999)

b) Transcription (Muchardt et al. 1998; Korneberg et al, 1995; Felsfenfeld et al, 1996; Svaren et al, 1996; Kadonaga et al, 1998)

c) Cell differentiation (De La Serna et al, 2001)

d) Recombination (Kwon et al.,2000, Roth et al.,2000, S tanhope-Baker et al, 1996) Shelter (Ura et al., 2001).

The nucleosome plays a dynamic role in the regulation, playing the role of in vivo gene repressor (Han et al, 1988).
This is due to the fact that the compaction of DNA sequences in highly ordered structures thus prevents the binding of specific protein factors to their target sequences of DNA.
The accessibility of the DNA enzyme is regulated by complex, coordinated with each other as the ATP-dependent remodeling complexes and histone modifying complexes.

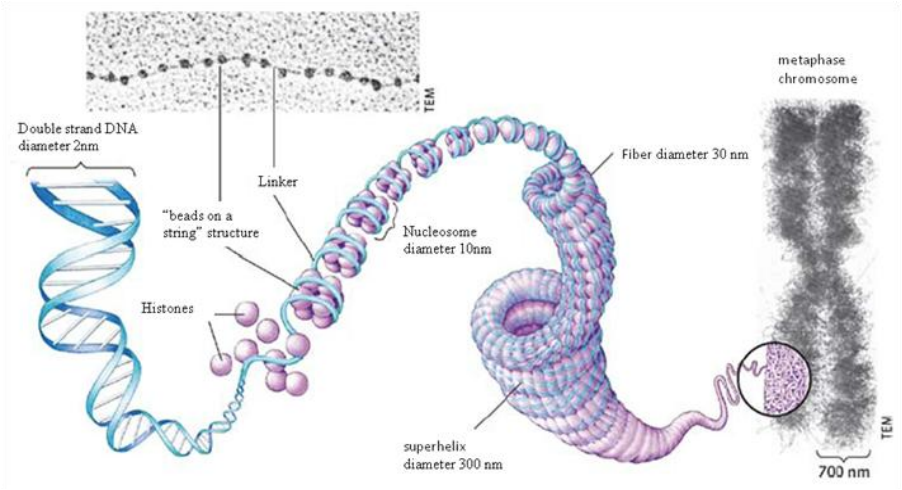


Fig. 1 Different chromatin structures that reflect higher order chromatin organization.

1.2 Post-translational modifications and histone code

A post-translational modification is the chemical modification of a protein after its translation. Different histone modifications works sequentially and in combination to built a real “histone code” (Strahl et al, 2000).

The changes are identified and characterized: methylation, acetylation, ADP – ribosylation, ubiquitinazion and phosphorylation. Considering the electrostatic components of the complex chromatin, acetylation which neutralized the positive charges, or phosphorylation wich adds negative charges, can initiate chromatin decondensation of the fiber.

The same type of modification, but of different amino acid residues, or combined with other types of changes can cause various forms of activation. For example acetylation of lysine K16 of histone H4 N-terminal tail is present during the transcriptional activation, whereas acetylation of K12 in the own tail is in heterochromatin regions (Allis et al., 1985). Acetylation is a reversible post-trandlational modification involves the transfer of an acetyl group to amino-teminal end of a conserved lysine residue.

The neutralization of positive charges of the histone tails is thought to reduce their affinity with DNA and cause changes in nucleosomal chromatin (Bauer et al.,1994; Garcia Ramirez et al., 1995). For a long time, acetylation has been associated qith transcriptionally active regions of chromatin, while deacetylation to the repression of several inducible genes.

Phosphorylation of proteins is one of the most important regulatory mechanisms. Many enzymes and receptor are switched on and off by phosphorylation and dephosphorylation events, through the action of specific kinases and phosphatises (protein appointed to the removal of a phosphate group from the protein).

Phosphorylation is a modification that is involved in the activation of transcription in meiotic chromosome condensation (Cheung et al., 2000).

The ubiquitination is a post-translational modification has emerged as a key element for the regulation of cellular processes.

This is a process that requires the establishment of a covalent bond between a molecule (or molecules) of ubiquitin and target proteins.

Mono-ubiquitination helps to alter the target protein or protein interaction of these with other factor, on the other hand the multi-ubiquitination leads to degradation of the target protein by the work of the proteasome (Varshavsky et al, 1997).

The specificity of the mechanism of ubiquitin is regulated by different combinations of enzymes E1 (ubiquitin-activating protein), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) (Fig. 2).

In the yeast hitone ubiquitination at lysine 123 has as its substrate histone H2B tail and Rad6 was identified as the enzyme that conjugates ubiquitin to histone.

The histone methylation has two targets, the arginine and lysine.

The histone methyltransferase (HMT) acting on arginine residues and are involved in activating gene, recruited as co-activators on the promoters.

The methylases acting on lysines are involved in gene silencing and possess a highly conserved SET domain. Set1 in yeast, homologous to the human gene and MML Tritorax in D. melanogaster, methylates Lys 4 of H3, and this methylation is associated with a stage of active transcription in chromatin (Litt et al., 2001). In the transcription is also involved in the Set2 methylase that acts on lysine 36 of H3.

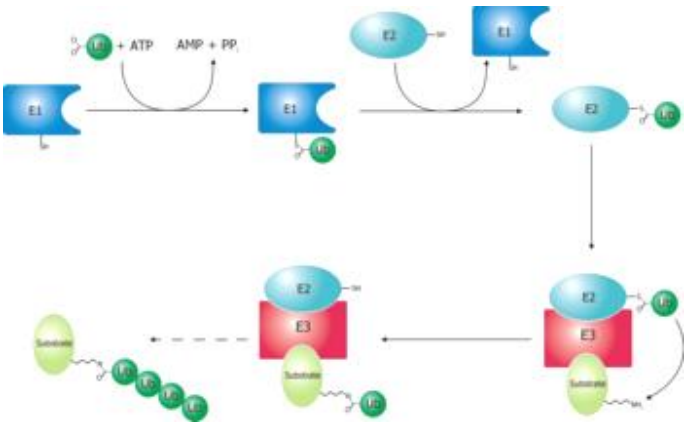


Fig. 2 Mechanism of ubiquitin is regulated by different combinations of enzymes E1 (ubiquitin-activating protein), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase).

Trascription

2.1 Eukaryotic transcription

The transcript consists of a series of biochemical events allow the passage of genetic information from DNA to RNA molecules, through the action of an enzyme, RNA polymerase, which incorporates the nucleotide tri-phosphates into an RNA chain for which the DNA strand acts as a mold. While prokaryotic cells possess only one type of polymerase responsible for the training of all mRNAs, eukaryotic cells have three different enzymes: RNA polymerase I which synthesizes ribosomal RNA (rRNA),

RNA polymerase II, which mainly produces the 'messenger RNA(mRNA) and RNA polymerase III which synthesizes Transfer RNA (tRNA), 5S and some small RNA molecules. For all the polymerase exist promoters and terminators which define the unit of transcription and regulate the speed and efficiency of their operation.

2.2 Regulation of transcription

The regulation of transcription of eukaryotic protein-coding genes is a complex and finely regulated process that requires concerted operation of many proteins and transcription factors.

These factors can be classified into three groups:

a. Transcriptional regulators binding specific DNA sequences, as activators and repressors, which bind to proximal promoter elements and/or distal regulatory sequences (enhancers and silencers) by modulating the level of transcription of specific target genes in a tissue-specific and development, or in response to physiological or environmental stimulation.

b. General transcription factors are ubiquitous and include RNA polymerase II (Pol II) and a series of accessory transcription factors of the general initiators (GFTs) that bind to the "core" elements of the promoters of DNA, and as the TATA box initiators, allowing the recruitment of Pol II on the specific promoter of class II genes.

c. Co-repressor and co-activators that interact with regulators mediating and facilitating the effect of those on the basal transcription machinery or through physical interactions with GTFs and / or Pol II or indirectly through changes in chromatin structure.

Cooperative protein-protein interactions and protein-DNA play an essential role in specific binding of activators on the enhancer, resulting in the formation of specific nucleoprotein complex called enhanceosom (Merika et al, 2001) and in the precise assembly and Pol II GTFs a set of pre-starting stable "core" of the promoter (Orphanides et al., 1996).

The general unit of transcription is composed of Polymerase II and six GTF proteins, including TFIIA (Transcriptional Factor IIA) TFIIB, TFIID, TFIIE, TFIIF and TFIIH. With the exception of TFIIB, a single polypeptide of 35 kDa, the other

components are stable multiprotein complex consisting of two to four different proteins. The Pol II itself is a multi-subunit enzyme, about 0,5MDa, consisting of twelve different proteins, five of which (RPB5, RPB6, RPB8 and RPB10, RPB12) shared with two other eukaryotic RNA Polymerase. The promoters of RNA polymerase II represent the clearest example existsregarding the relationship between chromatin structure and gene expression.

Eukaryotic gene expression is subject to multiple levels of control, but the start of transcription is undoubtedly one of the first and most important adjustment layers. For accurate transcription initiation is not required, however, only the RNApolymerase II, but also a number of other factors, some of which are shared by all RNA polymerases, acting on the structure of chromatin. The presence of nucleosomes along the promoter makes it inaccessible to regulatory sites on DNA by the transcription machinery, thereby inhibiting RNA polymerase II to perform its function. Inducible genes in eukaryotes can occur in two differente functional states depending on how the nucleosomes are arranged along the promoter region (Wallrath et al., 1994).

A first class contains genes predisposed in which the promoter regulatory regions are left free of nucleosomes

after th replication process. In this class of genes, induction of transcription occurs very rapidly as positive regulators can easily bind. The second class of genes that have reshaped the entire promoter region covered by nucleosomes, which must be disassembled to allow the binding of regulators. However in both cases the position of nucleosomes along the promoter region is critical for proper gene regulation.

Biogenesis of ribosomes in Saccharomyces cerevisiae.

Ribosomes are particle ribonucleoproteic controllers translation process, that is the transition from information contained in the DNA molecule to the function, represented by the proteins. Whithin eukaryotic cells the synthesis of ribosomes requires the coordinated expression of a complez series of steps that include:

a) Transcription of 5S RNA dependent RNA polymerase.

b) Association of 5S RNA with specific ribosomal proteins and their assembly into 60S subunits.

c) The expression of the precursor of the 35S rRNA by RNA polymerase I.

d) The processing of the precursor 35S by exonuleases, endonucleases, methylases, etc. Transcription of ribosomal protein genes by RNA polymerase II.

e) The processing of these transcripts by capping, splicing and polyadenylation of the transcript and the subsequent migration to the cytoplasm.

f) Translation of mRNA for ribosomal protein.

g) Transport to the nucleus of the ribosomal protein formed.

h) Assembly of these new proteins with the ribosomal RNA precursor protein and participation of its processing.

i) Ribosomal subunit to complete migration of the cytoplasm.

The expression of several ribosomal genes must be highly controlled in order to ensure equimolar production of rRNA and ribosomal proteins. Furthermore, the cells can modulate the expression of ribosomal genes in response to changes in growth conditions, such as changes in nutritional conditions, temperature etc, which alter the demands necessary for the biosynthesis.

The regulation of expression in yeast is carried out almost completely at the level of transcription (Raue et al., 1991).

In a yeast culture, 60% of the transcript is due to the production of rRNA and 50% transcript of RNA poplymerase II and 90% of the splicing are dedicated to the production of ribosomal proteins.

In addition, transcripts of 137 genes coding for ribosomal protein, 78 are the most abundant mRNA of the cell despite

their short half-life (Velculescu et al., 1997). The regulation of ribosome synthesis is a key process for the vitality of the cell. Despite the wide range of information on the structure and function of ribosomal genes in yeast, the regulatory mechanism that controls the expression of these genes is not yet fully clarified.

3.1 Ribosomal DNA

The ribosomal DNA (rDNA) is genomic region encoding the 35S ribosomal RNA precursor in *S.cerevisiae* and the 45S in the more complez eukaryotic. It consist of a highly repeated sequence which is not transcribed spacers NTS) separate the coding regions of rRNA. The spacers regions contain important regulatory saquences for transcription and replication. The promoter is recognized 35S rDNA by polymerase I specific transcriptionof this RNA. Enhancer elements are responsible for the high rate of transcription of RNA polymerase I. The rDNA proteins associated with the nascent rRNA chain to form their own division of the nucleus called the nucleolus.

3.2 Expression of ribosomal proteins genes

Yeast Ribosomes consist of 32 small subunit and 46 large subunit ribosomal protein encoded by 137 genes, 59 of them

are present in duplicate copy. Was not detected the presence of some pseudogenes, in contrast to what happens in mammalian cells, where there is the presence of a single functional gene and several pseudogenes (Dudov et al., 1984).

The two copies were probably formed as a result of a process of gene duplication; the ORF (open reading frames) of the duplicated genes are highly conserved while the sequences leaders revealed significant differences. Characteristic of ribosomal protein genes is the presence of an intron located 5 'of the coding region or, in some cases, in the leader sequence. The genes for ribosomal proteins are distributed throughout the genome and are transcribed independently. The cellular levels of mRNA of various ribosomal proteins are almost the same, and since their half-life is roughly similar, makes clear that the production of ribosomal proteins is regulated at the level of transcription. The majority of the promoters of these genes and the genes of the translation factors contain two binding sites for Rap1p and ten contain binding sites for Abf1p (Mager et al., 1991). Two promoters have also binding sites for the protein Reb1.

Abf1p and Rap1p are both involved in other cellular processes. Rap1p binds to sequences 5 'of many genes

including those encoding glycolytic enzymes highly transcribed, of conjugative type loci and telomers. Abf1p, Rap1p as seems to have the dual role of transcriptional activator orrepressor depending on the context in which it is inserted. RAP1 and Abf1 are proteins that bind to the DNA will cause a strong folding and create a region free of nucleosomes, which varies from 70 to 230 base pairs. These proteins play primarily a structural role by organizing the chromatin structure in order to allow other regulatory proteins to bind with their binding sites (Planta et al., 1995). This idea is supported by the finding that the binding sites for Abf1 can functionally replace the Rap1p-binding and back, both promoters of ribosomal genes in other promoters.

The promoters of ribosomal genes also have a region rich in T, which are ubiquitous on the promoters of genes transcribed by Pol II and their effect on the activation of transcription seems to be due to the peculiar structure of their poly dT / dA, which may prevent the binding of nucleosome to the promoter DNA.

Proteasome

4.1 The ubiquitin-proteasome system (UPS)

The proteasome is a multiprotein complex that is responsible for degrading polypeptides within the cell (Fig. 3). The intracellular proteolysis plays a crucial role in many cellular processes, such as cell cycle, signal transduction, gene expression, development, maintenance of protein folding, processing of antigens and other cellular processes (Kirschner et al., 1999).

Eukaryotic cells use two main mechanisms for protein degradation: lysosomal way, for those of membrane, and the ubiquitin-proteasome path, what is most used by eukaryotic cells for degradation of cytoplasmic proteins. The proteasome has a threefold function: it eliminates incorrectly folded protein, breaks down proteins into peptides with a length of three to twenty amino acid residues used for antigen presentation on MHC class I molecules (Rock et al.,1994) and finally degrades specifically the factors involved in cell cycle such as cyclins (in mitosis). Substrates are designated for destruction in this pathway by the covalent attachment of a polyubiquitin chain. The generation of such polyubiquitin chains requires the activity of three

classes of enzymes (Pickart, et al., 2004). A ubiquitin molecule is first activated for transfer via formation of a thiolester bond with the ubiquitin activating enzyme, or E1. This activated ubiquitin is then transferred to a ubiquitin conjugating enzyme, or E2, again via formation of a thiolester bond. Typically, the activated ubiquitin molecule is then transferred to the substrate in collaboration with a ubiquitin ligase, or E3. Two general paradigms for ubiquitin ligation exist. In the first, the E2 transfers the ubiquitin molecule to the E3, which possesses its own catalytic activity and is responsible for ultimate transfer of ubiquitin to substrate. In the second, the E3 functions non-catalytically with E2 to promote ubiquitin transfer. Ubiquitin itself is a highly conserved 76 amino acid protein which invariably ends in a diglycine motif. A covalent bond is formed between ubiquitin and substrate typically via the carboxyl group of the terminal glycine in ubiquitin and the ε-amino group of a lysine residue within the substrate. Less commonly, other nucleophiles may be modified by ubiquitination. (Hanna, et al., 2007). Polyubiquitin chain formation proceeds typically via isopeptide bond formation between the G76 carboxyl group of the “n+1” ubiquitin to the ε-amino group of a lysine within the preceding ubiquitin. Seven lysines are found in ubiquitin, allowing for the generation of a variety of

polyubiquitin chain types (Peng et al, 2003). The minimum length of a polyubiquitin chain capable of supporting degradation appears to be approximately four (Thrower et al, 2000), although many substrates appear to acquire polyubiquitin chains that are significantly longer. The fundamental role of the substrate-linked polyubiquitin chain is to serve as a recognition motif for a large multi-subunit protease known as the proteasome, which is responsible for hydrolyzing the substrate's peptide bonds, thereby reducing a folded protein into oligopeptides. Other proteases convert these peptide products into free amino acids, which can then be used a new in biosynthetic processes. The proteasome is an approximately 2.5-MDa protein complex thought to consist of at least 33 subunits in the budding yeast *S. cerevisiae* (Schmidt et al., 2005). The proteasome can be divided by biochemical methods into two smaller complexes (Fig. 1). The first is the 670 KDa core particle, also known as CP or the 20S particle. The CP is a barrel-shaped complex consisting of four stacked rings, each ring composed of seven proteins (Groll et al., 1997). The two outer rings are identical, as are the two inner rings, giving the CP an "αββα" symmetry along a vertical axis, as well as a sevenfold pseudo-symmetry along the horizontal axis. The peptidolytic active sites of the proteasome are sequestered within a

cavity formed at the center of the CP by the two inner (beta) rings (Groll et al., 1997). There are three distinct peptidolytic activities in the CP, each represented twice: a tryptic activity (i.e. cleaving after basic residues), a chymotryptic activity (i.e. cleaving after hydrophobic residues), and a post-acidic activity (Kisselev et al., 2006). On either side of the central cavity is a second cavity formed between an inner (beta) and an outer (alpha) ring, but the exact function of these outer chambers remains unknown. Substrates gain access to the CP only though narrow pores present at either axial end of the CP and structurally represented by the N-termini of alpha ring subunits (Groll M, et al., 2000). Importantly, the pores leading to the interior of the CP are shut in the basal state, and require a mechanism of gate opening to facilitate substrate degradation. This function, among others, is carried out by the second subcomplex of the proteasome: the regulatory particle, also known as the RP or 19S particle or PA700. The RP is a nearly 1-MDa complex consisting of at least 19 proteins. One RP may associate with either axial end of the CP. When the CP is associated with one or two RP species, the complex is referred to as the 26S proteasome. The RP may be further divided by biochemical means into two sub-complexes of its own, the base and the lid (Glickman et al., 1998). The base subcomplex is located

proximal to the CP and contains six AAA-type ATPases (Rpt1-6) as well as four non- ATPase subunits (Rpn1, Rpn2, Rpn10, and Rpn13). The six ATPases of the base are thought to form a ring, as well as to possess a protein remodeling capacity presumed to function in substrate unfolding (Braun et al., 1999; Liu CW et al., 2002). The pore leading into the CP is too narrow to be traversed by most folded proteins, and as such, unfolding is thought to be a prerequisite for the degradation of the majority of proteasome substrates. Despite very high sequence similarity among the six ATPases, early work demonstrated their non-equivalence of function (Rubin DM et al., 1998), and delineating their precise roles remains an active area of investigation. One ATPase, Rpt2, is known to play a role in opening the pore into the CP to facilitate substrate entry (Kohler A, et al., 2001), while Rpt5 is thought to play a role in recognition of the substrate-bound ubiquitin chain (Lam YA et al., 2002). Another base component, Rpn10, is clearly a ubiquitin receptor (Verma R et al., 2004; Elsasser S. et al., 2004). The functions of Rpn1 and Rpn2, which represent two of the largest proteins of the proteasome, have remained more elusive. Rpn1 is known to bind a series of ubiquitin chain receptors which are not core components of the proteasome, but rather, sub-stechiometrically-associating

proteins (Elsasser S. et al., 2002). The lid subcomplex of the RP is found distal to the base and was originally characterized as an eight-component complex with high sequence homology to two other cellular complexes, the COP9 signalosome and translation initiation factor eIF3 (Glickman MH, et al., 1998). All three complexes are characterized by two protein motifs known as the MPN and PCI domains. The lid possesses six PCI-domain-containing subunits (Rpn3, Rpn5-7, Rpn9, and Rpn12) and two MPN domain proteins (Rpn8 and Rpn11). The PCI domain remains poorly understood, but the MPN domain of Rpn11 is known to represent a metalloprotease-like deubiquitinating activity which removes ubiquitin from substrates (Verma R. et al., 2002). A corresponding activity in the COP9 signalosome removes the ubiquitin-like modifier Nedd8 from the cullin subunits of various protein complexes (Cope GA. et al., 2002). No such activity has yet been identified in eIF3. The MPN domain of Rpn8 is very similar to that of Rpn11, but lacks crucial catalytic residues. In recent years, a ninth member of the lid, Sem1 or Rpn15, has been identified.

4.2 UPS components in the cell nucleus

Strong evidence shows that the proteasome is localized both in the cytoplasm in the cell nucleus (Hugli et al., 1983; Kleinschmidt et al., 1983; Reits et al.,199 7; Adori et al., 2006).

The nuclear localization depends primarily on the cell type, density and growth conditions, and the relative concentrations of nuclear and cytoplasmic proteasome can vary between 17%and 50% (Rivett et al., 1992). It has been shoe that some subunits of the 20S proteasome possess nuclear localization signals (Nederlof et al., 1995) and phosphorylation of tyrosines present be borne in them play a a key role in the transfer of the proteasome form the cytoplasm ti the nucleus (Tanaka et al., 1990). In both the yeast 20S, 19S regulatory particle that appears to be imported into the nucleus as inactive precursors (Lehman et al.,2002, Wendler et al., 2004).

Instead, there is experimental evidence about the reverse process, ie the exportof the proteasome, or proteosomal subunits , the cell nucleus to the cytoplasm, so it could be transported in a unidirectional manner in the nucleus during interphase (Reits et al., 1997) and the pool

of proteasome cytoplasmic and nuclear power could be balanced during mitosis. The nucleus also contains other components of the UPS system, such as ubiquitin,the 19S regulatory complex (Peters et al., 1994), the ubiquitin specific protease Hauspie (Everett et al., 1997) and E1-E3 enzymes (Roth et al., 1998).

4.3 The nUPS system's role in gene expression

Although it is clear that proteasome components influence various stages in transcription, it is debated whether these components function as part of complete 26S proteasomes or whether they are acting as discrete sub-complexes, such as 19S, or even sub-sub-complexes, such as APIS (AAA proteins independent of 20S). Proteasome mutations that affect transcription are typically found within the 19S ATPases, and, indeed, early chromatin immunoprecipitation (ChIP) experiments could detect only 19S ATPases — and not 20S components — on promoter DNA in vivo (Gonzalez F,et al., 2002). More recent experiments, however, have shown that the proteolytic activity of the proteasome can be important for transcription (see below), and ChIP data from several groups have revealed the presence of 20S proteins at promoters (Morris MC. et al., 2003). The exact way in which proteasome subunits interact with chromatin is not

clear. 19S subunits are localized to promoters and coding regions of genes in yeast, although when they arrive is debated. At the GAL genes, for example, one group has reported that 19S components interact with chromatin only when the genes are active; another group has found that 19S proteins are localized to GAL promoters prior to activation, and they redistribute along the gene when transcription occurs, as if they are moving with RNA polymerase (Ezhkova E. et al., 2004). At least three mechanisms have been proposed to explain how proteasome subunits associate with chromatin. Monoubiquitylation of histone H2B is necessary for stable association of proteasomes with both active and inactive chromatin, although it is unclear how a single ubiquitin attached to H2B is able to recruit the proteasome. Another mechanism for proteasome recruitment is through specialized promoter-bound factors such as Cks1 (Cdc2 kinase subunit), which plays an essential role in recruitment of proteasomes to the Cdc20 (cell division cycle 20) gene in yeast (Baskerville, et al., 2005). Finally, because proteasome proteins can interact with both transcriptional activation domains (Chiari E. et al., 2004) and components of the general transcriptional machinery, it is possible that transcription complexes themselves recruit proteasome

components and pass them among various factors during different phases of the transcription process. The most striking activity of the proteasome is proteolysis. As the ultimate post-translational modification, proteolysis provides directionality and irreversibility to biological processes and drives changes in protein–protein interactions and complex assembly. One of the first realizations indicating that proteolysis is important for transcriptional regulation came from studies of pol II degradation in response to DNA damage. As part of the transcriptional-coupledDNA repair process, the elongating form of the largest subunit of RNA pol II is selectively targeted for ubiquitin-mediated proteolysis (Krogan NJ. et al., 2004; Somesh BP. et al., 2005). The destruction of ‘active’ pol II limits transcription until DNA damage can be repaired — thus protecting the integrity of the proteome — and selectively targets active genes for efficient and quick restoration. Proteolysis, however, is not just an emergency measure for transcription but is also a part of the transcription process itself. Many transcriptional activators are unstable proteins whose rate of destruction mirrors their ability to activate transcription (Reid G. et al., 2003). The exact nature of how activation and destruction are linked is not yet clear. One possibility is that transcription-coupled destruction of activators serves as a

limiting mechanism to micro-manage the amount of transcription that any single activator can stimulate (Tansey, et al., 2001). By having the destruction of transcription factors 'hard-wired' into the activation process, cells ensure that repeated rounds of transcription from a particular promoter require a constant supply of fresh activator. In some notable cases the proteolytic activity of the proteasome is required for transcription. Transcriptional activation by the progesterone receptor, for example, is inhibited by drugs that inhibit the proteasome, and this inhibition correlates with reduced recruitment of RNA pol II to genes regulated by the progesterone receptor (Dennis AP. et al., 2005). Similarly, blocking ubiquitin-mediated proteolysis of the yeast activator Gcn4 blocks the ability of Gcn4 to recruit RNA pol II to promoters. One area in which proteolysis might also prove important is in enabling transcription complexes to change their nature by swapping out one or more components. Often, activation of transcription requires that repressor complexes be replaced with activation complexes capable of recruiting RNA pol II. Events that occur after initiation of transcription might also require intervention of the ubiquitin–proteasome system. Ubiquitylation of Gal4, for example, is required so that Gal4-recruited, initiated RNA pol II complexes can acquire

appropriate phosphorylation signals needed for pre-mRNA processing. Finally, it is worth noting that transcription termination itself probably involves the proteasome, because the 30 ends of actively transcribed genes have been found to be enriched with 20S proteasomes, and inhibiting the proteasome blocks appropriate transcriptional termination (Gillette TG. et al., 2004). Ample genetic evidence suggests that 19S proteasome components, particularly the base ATPases, influence transcription through a non-proteolytic mechanism. It seems reasonable to conclude that, in this capacity, the 19S proteins are making the most of their chaperone functions (Liu C-w. et al., 2002) protein unfolding and protein translocation. Besides elongation, other aspects of transcription are also under the influence of 19S proteins. Methylation of histone H3, for example, which is a signal that defines active sites of transcription, is dependent on Rpt4 and Rpt6. We have suggested that 19S proteins might converge on sites of active transcription — in a way that depends on ubiquitylation of histone H2B — and somehow act on local chromatin structure to promote recruitment of appropriate methyltransferases or enable these methyltransferases to access their target sites on the histones. More recently, it has been found that the 19S complex can use the energy of ATP hydrolysis to drive

stable complex formation between Gal4 and the SAGA (Spt Ada Gcn5 acetyltransferase) complex, one of its essential targets in the transcriptional machinery, (Lee D.et al., 2005). Interestingly, SAGA recruitment by Gal4 is one of the very first steps in GAL gene activation, illustrating that 19S proteins can act at both early — pre-initiation recruitment of SAGA— and late—elongation —steps in the transcription process. The involvement of 19S ATPases in perhaps the earliest phase in GAL gene activation provides a simple explanation for why 19S proteins are present on GAL genes before induction and leads to a model in which these proteins might act as 'loading factors' to facilitate formation of pre-initiation complexes at sites of transcription in vitro much in the same way as the DNA replication factor Orc sits on origins of DNA replication throughout the cell cycle and uses the energy of ATP hydrolysis to drive the loading of MCM (mini-chromosome maintenance) proteins before initiation of DNA replication. An alternative possibility is that 19S might exploit its translocase ability to selectively remove one or more components from the SAGA complex components such as Spt8, which plays an inhibitory role in SAGA function (Warfield L. et al., 2004). Indeed, the ability of 19S to 'suck' out specific factors from a complex might have a more general role in controlling the composition of

complexes at promoters and regulating transcriptional activity.

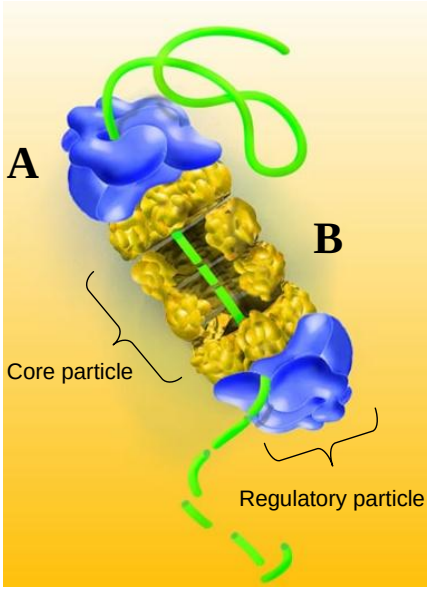


Fig. 3 The proteasome is a multiprotein complex that is responsible for degrading polypeptides within the cell.

CCR4/NOT complex

The Ccr4-Not complex, conserved from yeast to human and composed of nine core subunits in yeast, Not1-5p, Caf1p, Caf40p, Caf130p, and Ccr4p. It exists in at least two forms of 1.2 and 2 MDa that are conserved across the eukaryotic kingdom. The complex is a global regulator of gene expression that contributes to regulate several of the steps, ultimately leading to the stable production of a functional protein. Indeed, this complex, initially described as a transcriptional regulator, is important for the appropriate distribution of the general transcription factor TFIID on promoters across the genome (Lenssen et al., 2005) but also for the post-translational modification and transcriptional activity of some stress transcription factors (Lenssen et al., 2005, 2007). Furthermore, the Ccr4-Not complex contributes to mRNA degradation, since two of its subunits, Ccr4p and Caf1p, are the major yeast deadenylase. Finally, one subunit of the complex, Not4p, was identified as an E3 ligase (Albert et al., 2002), and a role for protein ubiquitination in vivo was demonstrated (Panasenko et al. 2006). The roles of the non-enzymatic subunits of the complex are largely not understood, except for Not1p, which is the scaffold of the complex (Maillet et al., 2000).

5.1 Structure of the proteins of the Ccr4-Not complex

Features of the proteins of the Ccr4-Not complex are described below:

Not1p: there are no known functional motifs within this protein, but there are two regions that are strikingly glutamine-rich (between amino acids 1009–1058 and 1294–1364). Not1p is thought to be a scaffold for the Ccr4-Not complex and is the only protein of the complex essential for yeast viability (Maillet et al., 2000).

Not2p: there are no recognizable functional motifs within Not2p. It contains two functional domains, a C-terminal region involved in the conserved Ccr4-Not complex function, and an N-terminal domain that can interact with the Ada2 protein, a component of co-activator Gcn5p histone acetyltransferase (HAT) complexes (Benson et al., 1998).

Not3p: The functional role of the putative coiled coils within this protein and the putative HR1 domain (1–81), thought to bind small rho G proteins and play a role in signal transduction, is unknown.

Not4p: a functional ring finger domain between amino acids 33–77 was revealed by the determination of the solution structure of the homologous N-terminal region of the human Not4 protein (CNOT4) by heteronuclear NMR (Hanzawa et al., 2001). The function of Not4p as an E3 ligase was

confirmed by showing in vitro ubiquitination by CNOT4 ((Albert et al., 2002) . A coiled coil (94–127), and a Pham:rrm motif (139– 277), thought to generally be diagnostic of an RNA binding protein, but that can also be found in a few single stranded DNA binding proteins, are additionally present in Not4p. Their functional importance has not been evaluated yet. The not4-1 allele codes for a truncated protein that carries all of these identifiable domains within Not4p.

Not5p: this protein is 44% identical in its N-terminal region to Not3p (1–150 of Not5p can be aligned with 1–148 of Not3p (Oberholzer, et al., 1998), and like Not3p contains putative coiled coil motifs (39–66 and 126–176). There is some redundancy between the Not3p and Not5p homologous domains, since cells lacking the N-terminal domain of Not5p, or Not3p, grow well at high temperature, whereas cells lacking both are temperature sensitive (Oberholzer and et al., 1998).

Ccr4p: this protein has a typical leucine rich repeat that is necessary for its association with Caf1p and the other components of the Ccr4-Not complex (Draper et al., 1994). It also displays two glutamine rich domains (15–45 and 190–229). The C-terminal domain of Ccr4p (495–837) shares homology to a Mg2þ-dependent DNase/phosphatase family of proteins (Dlakic, et al., 2000). This domain is important

for Ccr4p activities that are both RNA and single stranded DNA 30 –50 exonuclease activities, with a preference for 30 polyA substrates. Interestingly, this domain when overexpressed as a fusion to LexA can partially complement the non-fermentative growth defect of a cell lacking Ccr4p suggesting is might be an independent domain of the protein.

Caf1p: its glutamine-rich N-terminal region (17–126) is an extension that is absent from its putative mammalian homologues and is not necessary for function (Shimizu, et al., 1999). A putative RNase D domain (147–433), that contains non-canonical residues at three of the five residues involved in the formation of the catalytic site, includes a second glutamine-rich domain (352–370). A recombinant fragment covering this region displays nuclease activity in vitro (Daugeron, et al., 2001).

Caf40p: There is a DUF2 domain (143–344) that is present in many bacterial proteins of undefined function (Croft, et al., 2000), but its molecular function is unknown. No mutants have been isolated.

Caf130p: There is a putative transmembrane domain (65–84). No mutants have been isolated.

5.2 Ccr4-Not complex and his multiple functions

Evidence that multiple functions are mediated by the Ccr4-Not complex has been accumulating. For instance, the N-terminal domain of Not1p interacts with Dhh1p, Caf1p and Ccr4p, all involved in mRNA degradation. The not1-2 mutant over-expresses this domain of Not1p and is synthetically lethal with the deletion of the gene encoding Dhh1p, Caf1p or Ccr4p. In contrast, TFIID interacts with the C-terminal domain of Not1p, as do the other Not proteins, and not2, not4 and not5 mutants are synthetically lethal with many TAF mutant alleles (Deluen et al., 2002; Lemaire et al., 2000). Interestingly, deletion of DHH1 suppresses AT-resistance, a phenotype associated with mutations in the NOT genes, but over-expression of DHH1 suppresses mutations in CAF1. In contrast, deletion of SPT3 suppresses temperature sensitivity conferred by the not1-2 mutation, but does not suppress AT-resistance. These results are compatible with the idea that AT-resistance is a phenotype that results from an effect on mRNA degradation, to which Dhh1p, Caf1p and Ccr4p and the N-terminal region of Not1p, contribute. In contrast, temperature sensitivity is more likely to result from an effect on transcription, to which TFIID, SAGA and the C-terminal domain of Not1p contribute.

A recent study demonstrating that the Ccr4-Not complex contributes to regulation of Msn2/4p transcription by the Ras/cAMP pathway (Lenssen et al., 2002) provides the first evidence that the Ccr4-Not complex acts as a global regulator of gene expression that responds to specific physiological stimuli. Transcription by the redundant Msn2/ Msn4 factors, that mediate transcriptional activation in response to environmental stresses, and which is known to be under negative control of the cAMP-dependent protein kinase (PKA), is de-repressed in all ccr4-not mutants.

A role for the Ccr4-Not complex in regulation of gene expression according to glucose levels or stress, is suggested both by the stressed phenotype of exponentially growing mutant cells described above, and the observed modifications of Not3p, Not5p and Caf1p upon stress or glucose depletion. These modifications correlate with a described functional role for the Ccr4-Not complex at the diauxic shift. One study has demonstrated that Not4p is necessary for the cells to adapt to the use of a different carbon source when they approach the diauxic shift, in particular to grow on galactose (Deluen et al., 2002). Furthermore, Not1p dependent repression of target genes is activated at the diauxic shift (Lemaire et al., 2000). Finally, Caf1p and Ccr4p are required for non-fermentative gene

expression. The sum or our current knowledge suggests that the Ccr4-Not complex may be in different functional states in cells growing exponentially in glucose, and in cells after the diauxic shift. It seems likely that the Ccr4-Not complex will also be responding to physiological signals other than glucose depletion or stress. But in any event, the definition of at least one type of physiological signal that the Ccr4-Not complex might be responding to, allows us to try and define what functions might be regulated by the complex in response to this signal, and how this occurs.

5.3 Functional correlation between Not4 and Jhd2 proteins

Not4 Protein is an E3 ubiquitin ligase and a subunit of the CCR4-NOT complex, which has roles in transcription regulation, mRNA degradation, and post-transcriptional modifications; with Ubc4, ubiquitinates nascent polypeptide-associated complex subunits and histone demethyase Jhd2. A study published in 2006 (Laribee R. N., et al., 2007) shows the existence of a link between the proteasome, in particular the 19S subunit, and the *CCR4/NOT* complex in the selective regulation of H3K4 trimethylation. Not4 mutants show a significant reduction in levels of H3K4me3 that is

observed in strains with damage on the COMPASS or lower levels of of Set1 mRNA. So CCR4/NOT selectively regulates tri-methyl H3K4 without affecting the integrity of COMPASS.

In particular, the RING domain of Not4 mediates protein-protein interactions and acts as an E3ubiquitin ligase to specific substrates, among which could be found, only after further detailed studies, the *JHD2* gene product.

In order for the cell is always ready to generate an immediate response, depending on the stimuli it receives, it is necessary that the activities of Set1 methylase are balanced by the Jhd2 demethylase activity (Tu, et al., 2007) . The status of repression of a few genes must be modulated by removal of H3K4 tri-methylation, although the prevantly posttranscriptional regulation of Jhd2, may cost a lot of energy for the cell.

A very interesting study has shown that there is a strong correlation between functional Not4 and Jhd2 activity (Mersman et al., 2009). In particular it is seen that Jhd2 is controlled by a mechanism dependent ubiquitin and, specifically, by the E3 ubiquitin ligase Not4-acting by its RING finger domain. It was pointed out that in over-expressing Jhd2 strains, the level of H3K4me3 is decreased, while there are no effects on methylation in over-expressing Jhd2 that are deleted for the PHD domain, and this shows

that, in order to balance the activity of Set1, it is required not only an abundant presence of Jhd2 but also its recruitment to chromatin sites through the PHD domain (Mersman et al., 2009). The study also highlighted the role played by the proteasome. in fact, when protasome catalytic activity is inhibited by MG-132, there is a high accumulation of Jhd2 which does not depend on increased transcription. Under normal conditions, Jhd2 levels remain very low so that H3K4 tri-methylation is mantained. The model suggest that Jhd2 demethylase is modified post-translationally with the addition of a ubiquitin chain and thus is directed toward the proteasome, which degrades it. The poly-ubiquitination of Jhd2 corresponds with increased levels of tri-methyl H3K4 and occurs under specific physiological conditions. Indeed, in *ΔNot4* strains there is a decrease in H3K4me3 and there is a return to normal levels in Δ*Not4/ ΔJhd2* strains. No significant effects on mono-and di-methyl H3K4 are observed.. In Not4 ΔRING strains, ie strains carrying RING finger domain deletion, show loss of E3 ubiquitin-ligase activity with consequent increase in Jhd2 and loss of tri-methyl H3K4. This suggests that the activity of RING finger domain Not4 regulates both Jhd2 protein levels and tri-methyl H3K4 levels . The histone demethylase are expressed in multicellular organisms, on a temporary

and tissue-specific and their expression patterns can be altered by various causes. If the demethylases are missregulated or aberrantly expressed they can have devastating consequences as the manifestation of a cancer or defects in the development leading to mental retardation. In normal growing conditions, the balance favors an high level of tri-methyl H3K4 in genes constitutively active until.

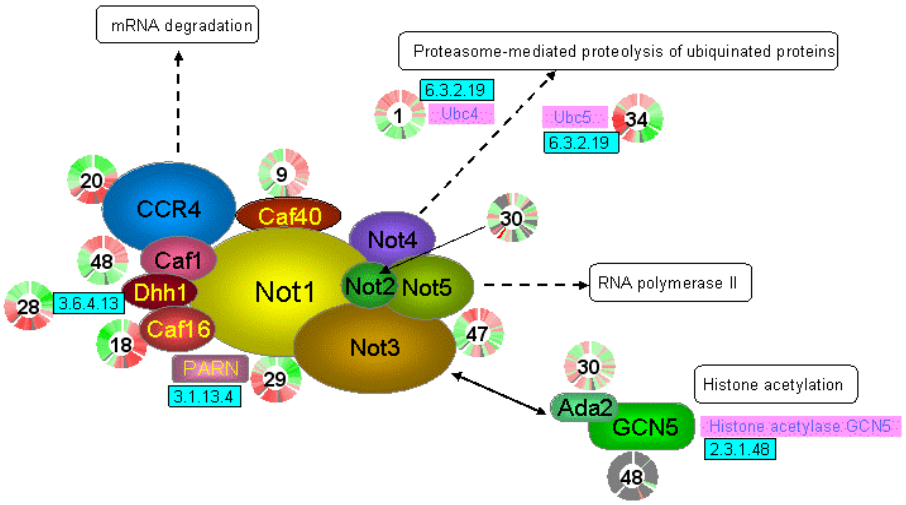


Fig. 4 The Ccr4-Not complex is composed of nine core subunits in yeast, Not1-5p, Caf1p, Caf40p, Caf130p, and Ccr4

AIM OF THE THESIS

Ribosomal protein genes are among the most active transcriptional units of RNA polymerase II. Their chromatin structure has unique features that are usually observed in highly transcribed genes such as: low level of histone occupancy in the promoter, high levels of H3K4 tri - methylation in the promoter and coding region, histone H2B ubiquitination; association of 19S and the 20S proteasome components with their chromatin . It is generally believed that maintenance of this features is essential in order to sustain a high rate of transcription. H3K4 tri-methylation is tightly coupled to H2B ubiquitination and progression of transcription through a complex network of interactions known as trans-tail path which also involves H3K36 and K79 methylation and CTD phosphorylation of RNA polymerase. Recent work suggests that H3K4 methylation activity of Set1 may be counterbalanced by the activity of demethylase Jhd2. Jhd2 demethylase has been shown to be locally targeted to proteolysis through ubiquitination by Not4 and Ubc4 proteins . Consistent with this interpretation, Not4 was previously shown to be required for efficient proteasome association and high levels of global H3K4 tri-methylation . When I started my thesis work it was unclear how and in which circumstances is the action of Not4 on Jhd2

controlled and what could be its biological function. The purpose of this thesis was to study the role of Not4 in determining the state of histone H3K4 methylation at ribosomal protein genes transcription units both in normal growth and after repression by Rapamycin and to understand its biological implications. The basic questions to answer were: Does Not4 and/or Jhd2 associate with RP genes? Does H3K4 methylation state at RP genes depend from Not4 action? In case of clear effects of Not4 on H3K4 methylation, do they depend on proteasomal function? Which is the effect of Not4 and Jhd2 on RP genes transcription? I believe that this thesis work gave a partial answer to these questions.

MATERIALS AND METHODS

Yeast strains

BY4741 MATa; his3Δ1; leu2Δ□; met15Δ0; ura3Δ0 Euroscarf

BY4742 MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0 Euroscarf

Not4-Tap S288C; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; Open biosystem

Pre1-Tap S288C; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YER012W::TAP Open biosystem

Rpn11-Tap S288C; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; YFR004W::TAP

Rpb3-Tap S288C; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YIL021W::TAP

Δ**bre1 BY4742**; Mata; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0 YDL074c::kanMX4 Euroscarf

Δ**bre1/Pre1-Tap** MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 YDL074c::kanMX4; YER012W::TAP This Study

Δ**bre1/Rpb3-Tap** MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 YDL074c::kanMX4; YIL021W::TAP This Study

Δ**not4 BY4742**; MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0 YER068w::kanMX4 Euroscarf

Δ**not4/Pre1-Tap BY4742**; MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0 YER068w::kanMX4; YER012W::TAP This Study

CFK920 W303; Mata, hta1-htb1::LEU2, hta2-htb2, ura 3-1, trp 1-1, leu 2-3, -112, his 3-11, ade2-1, can 1-100, GAPDH-HA-UBI4::URA3 M.A. Osley

CFK921 W303; Mata, hta1-htb1::LEU2, hta2-htb2, ura 3-1, trp 1-1, leu 2-3, -112, his 3-11, ade2-1, can 1-100, GAPDH-HA-UBI4::URA3 M.A. Osley

SDBY1066 BY4741: MATα his3Δ leu2Δ0 LYS2 met15Δ0 ura3Δ0 not4□::KanMX jhd2Δ::HygMX Mersman,Briggs

MBY1282 MATα his3Δ200 ade2::hisG leu2Δ0 ura3Δ0 met15Δ0 trp1□63 Ty1his3AI-236, Ty1ade2AI-515, SET1-N-3XMYC Mersman,Briggs

SDBY1073 as MBY1282 with pDPM4 Mersman,Briggs

Conditions of cell growth and treatments

Yeast cells were grown in YPD (2% Bacto-peptone 1% Bacto-yeast exstract, 3% Glucose) at 30°C. Cells were treated with 6-aza-uracil 2mg/ml for 20 minutes and with Rapamycin at final concentration 300nM for 60 minutes.

RNA Extraction, Analysis and Northern Blot

RNA was extracted from cell cultures at a density of 0.5-0.6 OD_{600}/ml or otherwise indicated. Cells were suspended in 1 ml of AE buffer (50 mM sodium acetate pH 5, 10 mM EDTA) centrifuged and resuspended in 0.4 ml of AE buffer plus 1%

(w/v) SDS. Cells were lysed with phenol:chloroform (5:1 pH 4.7), heated at 65 °C for 4 minutes, transferred at −80 °C for 10 minutes and aqueous phase was separated by centrifugation. After a second extraction with phenol:chloroform (24:1 pH5.2), RNA was precipitated with ethanol, dried and suspended in sterile water. Aliquots of RNAs (10 µg) were resuspended in 50% (v/v) formamide, 2.2 M formaldehyde and MOPS buffer (0.56% MOPS, 5 mM sodium citrate, 1 mM EDTA), heated at 65 °C for 15 minutes and loaded onto a formaldehyde/agarose gel (1% w/v agarose, 50 mM NaCl, 4 mM EDTA). After electrophoresis, RNAs were transferred to nylon filters (Biobond-Plus™ Nylon transfer membrane cod. N4781 Sigma-Aldrich) following the Northern blot procedure. Filters were hybridized with ^{32}P-labelled probes obtained from PCR amplification primers:

Oligonucleotides used for produce Northern Blot experiments probes:

Rpl30	Fw 5'-TACATTTCAACAGGCCCCAG-3'
	Rv 5'- CCACCTTGGAAGTAGTAGAC-3'
Rpl11a	Fw 5'-TCACGTTACCGTCAGAGGTC-3'
	Rv 5'-CACCTGGTCTGTTCATGACG-3'

Real Time PCR Protocol:

The DNA obtained from Chip and cDNA synthesis was quantified by reaction quantitative Real Time PCR (Applied Biosystems 7300). The amplification reaction was carried out by incubating the DNA in 30 µl using 15 µl of SensiMix SYBR HI-Rox Kit (Bioline Cat. Num. QT605-02), containing the enzyme, the deoxynucleotide triphosphate, SYBR Green (fluorophore for quantification of DNA double-stranded) and were added 12 µl of the pair of primers (200 pM / l). The reaction is carried out using as calibrator the amplified product from primer pair specific for the genomic region of the centromere (CEN3), telomere (TEL) or Peptidyl-prolyl cis-trans isomerase (CPR2). In separate wells were incubated aliquots of 3 l of DNA solutions of immunoprecipitated cell strains By4742 (representing mock), NOT4, PRE1, JHD2 and RPB1 and INPUT. The samples are subjected to a cycle that consists of 3 minutes at 95 ° C, 45 cycles of 15 seconds at 95 ° C and 1 min at 60 ° C (Protocol Bioline).

First-Strand cDNA Synthesis Protocol:

The following 20-µl reaction volume were used for 10 pg–5 µg of total RNA or 10 pg–500 ng of mRNA. The following components were added to a nuclease-free microcentrifuge

tube: 1 µl of oligo(dT)20 (50 µM); 5 µg total RNA or 10 pg–500 ng mRNA; 1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH). Sterile, distilled water to 13 µl. The mixture was heated to 65°C for 5 minutes and was incubated on ice for at least 1 minute. The contents of the tube was collected by brief centrifugation and were added: 4 µl 5X First-Strand Buffer; 1 µl 0.1 M DTT; 1 µl of SuperScript™ III RT (Invitrogen Cat. Num. 18080-044 200 units/µl). Next were mixed by pipetting gently up and down. The mixture was incubated at 42°C for 30-60 minutes. The reaction was inactivated by heating at 70°C for 15 minutes. The cDNA obtained was used as a template for amplification in Real Time PCR.

Oligonucleotides used for Real Time Experiments

Rpl24 orf1	Fw 5'-CAAAATCTACCCAGGCAGAGGT-3'
	Rv 5'-GAGGCAGATTTGGAGTTTTGGA-3'
Rpl24 orf2	Fw 5'-CAATTACCGGTGCTTCTTTGGA-3'
	Rv 5'-TGGCCTTCAATTTTTCTTCTCTG-3'
Rpl30 Prom1	Fw 5'-TCTGTGTGGTATTTCCTCCCG-3'
	Rv 5'-AGTCTCTCCAGGCAGGACGA-3'
Rpl30 Prom2	Fw5'-AGATGTAACGTTCCAAAATGTATGGA3'
	Rv 5'-TTTAAATGCGGCCCTAGCTG-3'
Rpl30 orf1	Fw 5'-TTCATGGGATTAGCAAGAAGGC-3'

	Rv5’-CAAACACAAGAGCAGGAATAAACTTT3’
Rpl30 orf2	Fw 5’-ATTTCAACAGGCCCCAGTTAAAT-3’
	Rv 5’-TACCAGACTTGATAACCAAAGCCA-3’
Rpl30 orf3	Fw 5’-GAATTGGGTACTGCTGTCGGTAA-3’
	Rv5’-TCAGAGTCACCAGCTTCCAAAA-3’
Rps12 Prom1	Fw 5’-CGCAGCCAAAGTTTTATACGAA-3’
	Rv 5’-CCGCTAGCCTGTATTTCATCCT-3’
Rps12 Prom2	Fw 5’-ACGAGGTTTGTACATCTTGGCA-3’
	Rv 5’-CAAATGTACGGATGCACAATGG-3’
Rps12 orf1	Fw 5’-AAGGTTTGGCTAACGACCCAG-3’
	Rv 5’-GATCTTACCCAAACCAGCCCA-3’
Rps12 orf2	Fw 5’-TGCAGTTGGCGAATACTACCTG-3’
	Rv 5’-AGGATGGATGTTCATGGCGTA-3’
Rpl21b Prom1	Fw 5’-TTCGACTAGGAGGTGAGG-3’
	Rv 5’-GTGTACAATCAAGCTCACCCGA-3’
Rpl21b Prom2	Fw 5’-GAGCGCGCAATATCGGTG-3’
	Rv 5’-TTTGCCTTTAACGCAATACAGTTG-3’
Rpl21b orf1	Fw 5’-TGGTGCCGTTCACATGTCC-3’
	Rv 5’-CATACCCTTTTGGATAGAACCATTG-3’
Rpl21b orf2	Fw 5’-AGGCCCAAGGTGTTGCTG-3’
	Rv 5’-AGGTTTCGTATGGAACTGGAGC-3’
Rpl17a Prom1	Fw 5’-TTCTGGCGAACGCATAGCT-3’
	Rv 5’-CTCACCATAATGATCACGACGG-3’
Rpl17a Prom2	Fw 5’-TAGCTCTATGGGCGCAGACA-3’

	Rv 5'-TTTTATGCTAGCCGTTGAAGCTC-3'
Rpl17a orf1	Fw 5'-CGCTGCCAACGCTGAAGTAT-3'
	Rv 5'-AATGGATTTTCCGCGCATT-3'
Rpl17a orf2	Fw 5'-CATATCCAAGTTAACCAAGCTCCAA-3'
	Rv 5'-TGTGGGATGGAGAAGATTCGTACT-3'
Cen3	Fw 5'-TTTAGCCAGAGAGGACTCGTT-3'
	Rv 5'-GGGTGGGAAACTGAAGAAAT-3'
TelV	Fw 5'-AAGTAGTCCAGCCGCTTGTT -3'
	Rv 5'-AGCGTAACAAAGCCATAATGC -3'
CPR2	Fw 5'-CGATAGTCTGCCCTTGCGAT -3'
	Rv 5'-CGTTGGTGGCAAATCCATCT -3'

Tap Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed essentially as described previously (18) with some modifications. Briefly, 100 mL of cells for each treatment at 0.4-0.5 OD_{600}/mL were cross-linked with formaldehyde at a final concentration of 1%. A cross-link time of 20 min at 30 °C was allowed before adding glycine to a final concentration of 330 mM. Cell pellets were resuspended in 400 µL of Lysis Buffer Low Salt (50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) supplemented with fresh protease inhibitor cocktail (Sigma) and lysed by vortexing with glass beads for 30min at 4°C.

Chromatin was sonicated to obtain fragments of average length around 400 bp. After immuno-precipitation with IgG Sepharose beads (IgG Sepharose™ 6 FastFlow cod. 17-0969-01 GE Healthcare), washing and elution, the cross-links were reversed at 65 °C, and DNA was purified.

To ensure that the enrichment obtained was specific to the TAP-tagged factors, the immuno-precipitated DNA from the untagged cells was systematically analyzed in parallel.

Regions were amplified from ChIP and input control samples using Power SYBR® Green PCR Master Mix (Applied Biosystems) in an Applied Biosystems 7300 Real-Time PCR apparatus, according to the manufacturer's instructions.

Relative quantification was carried out with the delta-delta-CT method (Applied Biosystems 7300 System SDS Software version 1.2). The peri-centromeric region of chromosome 3 (CEN3) was used as transcription state-invariant endogenous control.

Chromatin double immunoprecipitation (ChDIP)

ChIP experiments were performed essentially as described previously (19) with some modifications. Briefly, 100mL of cells for each treatment at 0.5-0.7 OD_{600}/mL were cross-linked with formaldehyde at a final concentration of 1%. A cross-link time of 20 min at 30 °C was allowed before adding

glycine to a final concentration of 330 mM and the cells were collected by centrifugation and washed two times with ice-cold PBS (137mM NaCl, 2.7 mM KCl, 100 mM Na2HPO4, 2mM KH2PO4). After the cell pellets were resuspended in 1000 µL of FA lysis buffer (50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 sodium deoxycholate, 1 mM PMSF) supplemented with a fresh protease inhibitor cocktail (Sigma), and lysed by vortexing with glass beads for 30min at 4°C. Chromatin was sonicated to obtain fragments of an average length of 400 bp. Following centrifugation at 13.000 g for 15 min at 4° C, the solubilized chromatin was transferred to a fresh tube, and the volume was adjusted to 1 mL with FA lysis buffer. For analysis of input chromatin, 20µL were removed. For the first immuno-precipitation, M2 agarose beads (Sigma) equilibrated in FA lysis buffer were balanced and incubated with the chromatin overnight at 4°C, the beads were collected by centrifugation and washed four times with FA lysis buffer and the immune complexes were eluted with 500 µL FA lysis buffer containing 200 µg/mL 3× Flag peptide (Sigma) overnight at 4°C. One-tenth of the eluate was reserved for “input”, and anti-HA antibody (12C5A, Roche) was added to a final concentration of 15 µg/mL to the remaining eluate, followed by incubation at 4°C overnight.

The immune complexes were collected by incubation with 40 µL protein G sepharose beads (Amersham) for 90 min. at 4°C and the beads were washed three times with FA lysis buffer and protease inhibitors, one time with 50mM Tris (pH 8).

The beads were resuspended in 50µL TE (pH 8) with 20 µg RnaseA (Sigma) and incubated at 37°C for 30 min.

Following a wash with 1 mL TE, the immune complexes were eluted from the beads by sequential incubation in 500 µL of 1% SDS/50 mM Tris (pH 8) for 5 min at 100°C and for 10 min at 65°C. After addition of 300 µL of NaCl Eluition Buffer concentration in each sample, the cross-links were reversed by incubation at 65°C overnight. DNA was entracted and resuspended in 100 µL TE (“input”) or 25 µl TE (“IP”).

Regions were amplified from ChIP and input control samples using Power SYBR® Green PCR Master Mix (Applied Biosystems) in an Applied Biosystems 7300 Real-Time PCR apparatus according to the manufacturer's instructions. The peri-centromeric region of chromosome 3 (CEN3) was used as transcription state-invariant endogenous control.

Relative quantification was carried out with the delta-delta-CT method (Applied Biosystems 7300 System SDS Software version 1.2).

Histone Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed and quantitated as previously described (18) with some modifications. In brief, yeast cells (100 ml) were grown in YPD medium to an OD600 of 0.6 and were cross- linked with 1% formaldehyde for 30 min before chromatin was extracted. The chromatin was sonicated (20 cycles, 60 s on/off, high setting) to yield an average DNA fragment of 500 bp. H3 antibodies (anti-H3 Abcam, rabbit polyclonal), H3K4me3 antibodies (Cell Signalling, rabbit monoclonal) and H3K4me2 antibodies (Abcam, rabbit monoclonal) were coupled to 20µl of protein-Agarose, previously equilibrated in Wash buffer (10mM Tris-HCl pH8, 0,25M LiCl, 0,5% NP-40, 0,5% sodium deoxycholate, 1mM EDTA). After reversal of the crosslinking and DNA purification, the immunoprecipitated and input DNA was analyzed by quantitative real-time PCR.

A coding region (*CPR2*) which has an histone enrichment around the genome distribution's median has been used as positive endogenous control for H3 occupancy.

A telomeric region from chromosome V (TELV) was used as endogenous negative control in di-methyl and tri-methyl-H3K4 determination.

A centromeric region (CEN3) was used as endogenous negative control in all other Chip determination.

Facs analysis

The cells are preinoculate in 20 ml of complete growth medium YPD and leave overnight at 30 ° C in agitation. The cells are then diluted in a volume of YPD medium with a target of 0.3 OD and grown until the exponential phase to 0.6 OD.. The pellet obtained is then washed twice in PBS1X. The samples to be devoted to reading in the FACS fluorescence are centrifuged at 3000 rpm for 3 minutes at 4 ° C and stored in the form of precipitation in dry ice at - 80 ° C prior 'to FACS analysis.
The evaluation of the cell cycle by FACS reading requires a process of fixation and staining of DNA with propidium iodide. For this purpose, samples were centrifuged at 3000 rpm at 4 ° C. Initially the cells are fixed, resuspending with 1ml of fixation buffer (250 mm Tris HCl pH 7.4, 70% EtOH) for two hours.
Following centrifugation at 3000 rpm for 3 minutes. Pellets are added to 0.5 ml of Tris HClpH 7.8 5omM, 0.5 mg of RNase A, 0.15 ug 5M NaCl and left to incubate at 37 ° Cfor 7h. After the 'elimination of RNase, the precipitates are resuspended in 1 ml of FACS buffer (Tris HCl pH 7.5 200 mm, 211 mM NaCl, 78 mM MgCl 2) and then again centrifuged and resuspended in 0.5 ml of FACS buffer with propidium

iodide 0.055 mg / ml. The samples are incubated for 12 hours at 4 ° C protected from light, the cells are sonicated for 2 seconds to 10% and read at Facs.

RESULTS

An active chromatin structure is partially maintained at RP genes after rapamycin repression.

Is well known that the transcription of genes for ribosomal proteins is highly active, so I wanted to test the effect of rapamycin on the level of histone occupancy, ubiquitination of H2B and H3 methylation of these genes. Rapamycin is an inhibitor of transcription of genes involved in ribosomal synthesis; this inhibitory effect is the result of blockage of the TOR (Target of rapamicyn) regulatory circuit. The locus object of my study is a typical RP transcriptional units coding for *RPL24A* and *RPL30* which has been extensively analyzed (Zhao et al., 2006). To map the exact localization of regulatory proteins, RNA polymerase and histone modifications in this locus , I designed 7 pairs of primers that cover the entire region encompassing the divergent promoter and both ORFs.

Firstly, I tested the efficiency of rapamycin inhibition (Fig1 panels A and B) by Northern blot analysis and ChIP localization of the Rpb3 subunit of Polymerase II . From the data reported in the figure it is clear that after 1 hour of treatment, transcription and localization of RNA polymerase are no longer detectable. Therefore I chose this

time-point as a reference for subsequent experiments. Fig 1, Panel C shows the levels of tri-methyl H3K4 in normal growth and after repression by rapamycin normalized to H3 histone occupancy on the same locus (shown in Fig.7, panel A). As it is known (Lee et al., 2005), in the very active RP genes during normal growth, histone occupancy is strongly reduced at the promoter and ORFs, as compared with a control amplicon, CPR2, which has an average histone occupancy. Treatment with rapamycin results in a slight increase in H3 occupancy in the whole region. Fig.1, Panel C, shows that after one hour of rapamycin repression, the normalized level of H3K4 tri-methylation is substantially maintained constant.

Histone H2B shows a distribution pattern in the *RPL24A/RPL30* locus very similar to that one observed for H3 (not shown). Since H2B ubiquitylation has been shown to be linked to H3 methylation (Weake et al., 2008), I tested the level of H2B ubiquitylation on the transcription unit in normal growth and after repression. In order to analyze the pattern of H2B ubiquitylation, I used a strain (*CK920)* carrying a flag on the HTB1 gene and a HA-tag fused to ubiquitin. The strain used as control for the background of the assay (*CK921),* carried a K123R mutation, preventing H2B ubiquitylation. I performed a chromatin double

immunoprecipitation (chDIP) (Fleming,A.B et al., 2008) and determined the relative level of H2B ubiquitylation in both strains during full rate transcription and after rapamycin repression. Fig.1, panel D shows that H2B is efficiently ubiquitylated in particular at the coding regions during full rate transcription. As it is evident, rapamycin repression causes a complete loss of H2B ubiquitylation in the whole region.. To conclude this first assessment of chromatin modifications, I tested the level of H3K4 di-methylation of *RPL24A/RPL30* transcription unit. Coherently with what observed for the H3K4 tri-methylation level, no increase of H3 di-methylation is detected in the whole *RPL24A/RPL30* transcription unit upon repression (Fig. 1, panel E).

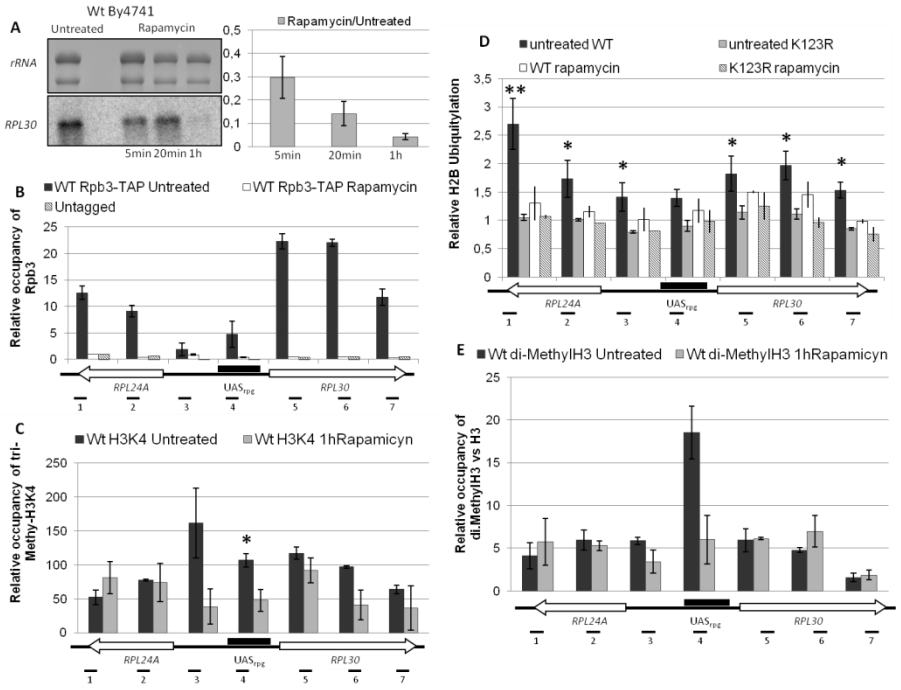


Fig. 1 Panel A: Northern blot showing *RPL30* mRNA accumulation in normal growth and at different times from addition of rapamycin to the medium. Panel B: Relative occupancy of RNAPII at *RPL24A/RPL30* transcription unit in normal growth and 1h after rapamycin addition to the medium. Panel C: Relative histone H3K4 tri-methylation during normal growth or in repressed condition. Panel D: Histone H2B ubiquitylation on the *RPL24A/RPL30* transcription unit. Panel E: Relative histone H3K4 di-methylation during normal growth or in repressed condition.

Not4 is required to maintain an H3K4 prevalently tri-metylated

It has previously been shown (Mersman et al., 2009) that Not4 E3 ligase, subunit Ccr4-Not complex, is involved in regulating of H3K4 methylation by ubiquitination of an histone demethylase, called Jhd2. Ubiquitination of Jhd2 by Not4/Ubc4 and subsequent proteolysis, represents a mechanism for controlling the levels of histone H3 methylation in yeast. So I tested the effect of the genetic background which regulates Jhd2 proteolysis on the levels of trimethyl- and dimethyl-H3K4, in standard growth and as a result of repression by rapamycin. Fig 2, Panel A shows the normalized level of tri-methyl H3K4 in a *Δnot4* strain which is impaired in Jhd2 proteolysis. Unlike in the wild type, where the levels of tri-methyl H3K4, following the addition of rapamycin to the medium, were only slightly lower than in standard growth conditions, in the ,*Δnot4* strain, H3K4 tri-methylation drops considerably after 1h repression.. Coherently in panel B, contrary to what was observed for the wild type strain, levels of H3K4 di-methylation normalized to the total level of H3, significantly increased after the addition of rapamycin to the culture medium. This is consistent with the role of Not4 in Jhd2

ubiquitylation and degradation. When Not4 is absent, the demethylase is not degraded and thus actively converts tri-methyl H3K4 in the dimethyl form. Consequently, the ratio tri-methyl/dimethyl-H3 in the *Δnot4* train, after repression, is in average almost ten times lower in the whole region, as compared with the wild-type strain (Fig. 2, panel C).

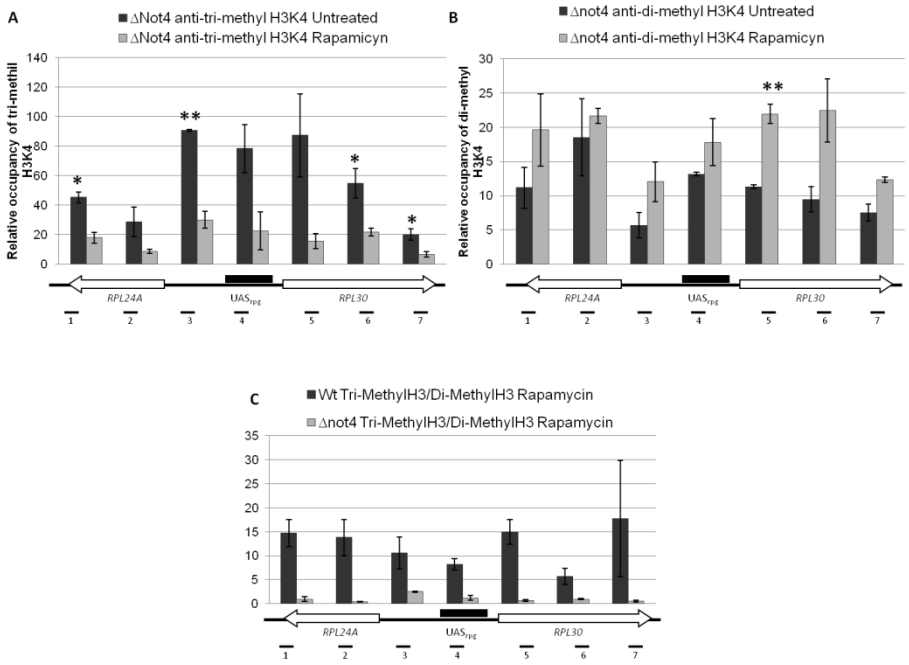


Fig.2 Panel A: Relative histone H3K4 tri-methylation in the delta *not4* strain during normal growth or in repressed condition. Panel B: Relative histone H3K4 di-methylation in the delta *not4* strain during normal growth or in repressed

condition. Panel C: Average ratio between tri-methyl and di-methyl H3K4 at 1h from rapamycin addition.

Not4 and Jhd2 associate with RP genes chromatin

It has never been directly demonstrated that Not4 acts directly on chromatin, so I studied its association with the *RPL24A/RPL30* chromatin by ChIP (chromatin immunoprecipitation) analysis. For this purpose I used a yeast strain in which the Not4 protein is fused to a TAP-tag sequence. Fig 3 shows the association pattern as obtained by ChIP. During normal growth Not4 association is generally low on the whole region (Fig.3a). The protein does not show a clear peak of occupancy, probably due to its prevalent association with nascent RNA or with transcription elongation complexes (Kruk et al., 2011)..

However, following treatment with rapamycin , Not4 localization significantly increases especially at the promoter and in the proximal portion of the of *RPL30* coding region .

In Fig 3a are also reported data of Not4 localization on three different transcription units (*RPL21b*, *RPS12* and *RPL17a*) which also encode ribosomal proteins. Also in these cases it is evident that Not4 occupancy increases at

promoters (and decreases at coding regions) upon repression.

From this set of experiments we can conclude that Not4 is present on RP genes transcription units and is ubiquitous during normal growth, while is predominantly localized at the promoter and proximal ORF segments under transcription repression by rapamycin.

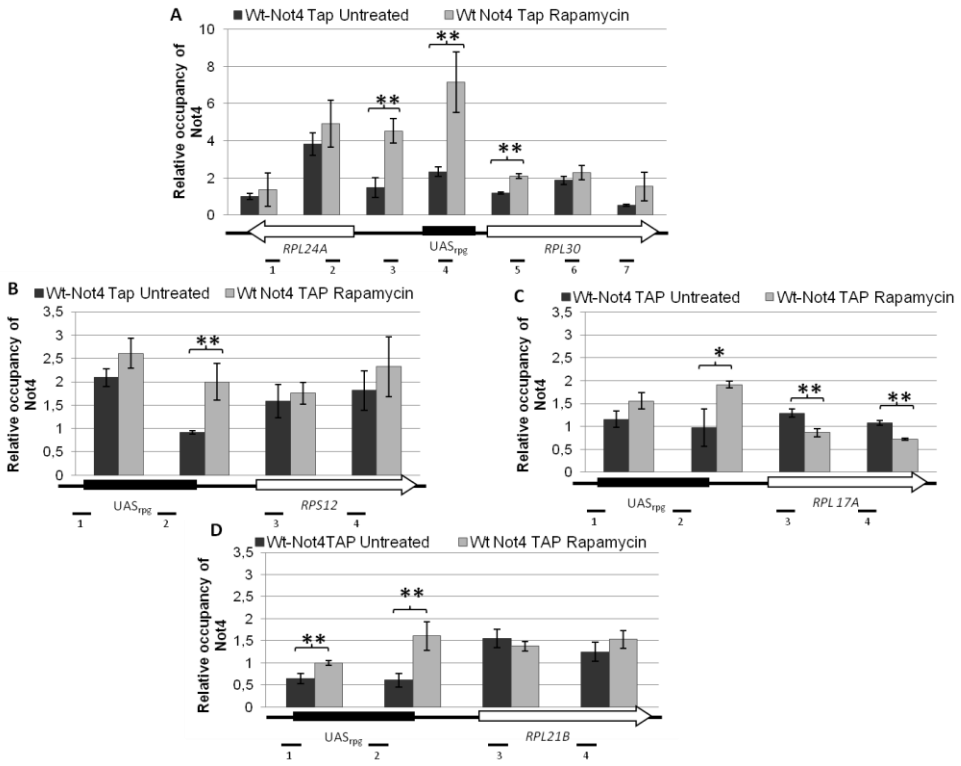


Fig. 3a Panel A: Relative occupancy of Not4 at RPL24A/RPL30 transcription unit during normal growth or after the addition of rapamycinin. Panel B: Relative occupancy of Not4 at RPS12 transcription unit in different conditions of transcription efficiency. Panel C: Relative occupancy of Not4 at RPL17A transcription unit in different conditions of transcription efficiency. Panel D: Relative occupancy of Not4 at RPL21B transcription unit in different conditions of transcription efficiency.

Next, I tested Jhd2 association with *RPL24A/RPL30* transcription unit both in wild type and in *Δnot4* background. The results (Fig.3b) show that, in the wild type background and in condition of active transcription, the demethylase associates with the promoter and proximal portion of *RPL30* ORF. This association is somehow reduced but still clearly detectable after rapamycin repression (Fig.3b). In the *Δnot4* strain there is a slight but significant increase in Jhd2 association both during active transcription and after rapamycin repression, coherent with the role of Not4 in Jhd2 degradation.

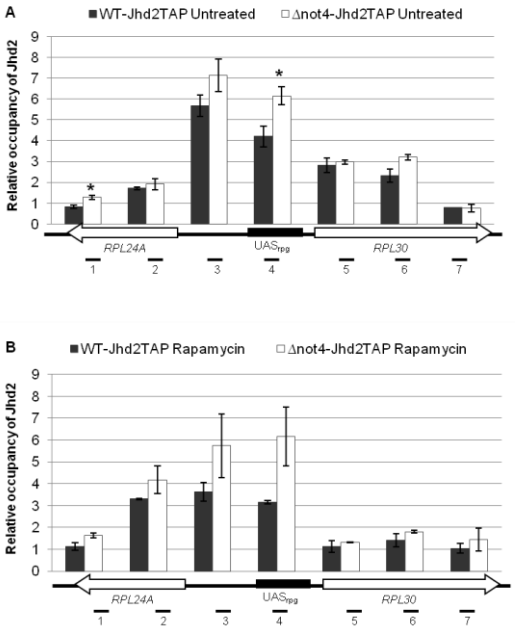


Fig. 3b Relative occupancy of Jhd2 at RPL24A/RPL30 transcription unit during normal growth or after the addition of rapamycin.

Not4 is required for efficient transcriptional reactivation of RP genes after rapamycin repression

Until now the data presented suggest that the association of Not4 on the promoters of genes for ribosomal proteins is increased as a result of repression by rapamycin and that its presence is necessary to maintain high levels of H3K4 tri-methylation. The question at this point is whether this local effect of Not4 may reflect a role in regulation of RP genes

transcription. In this regard, I analyzed in kinetics the recovery of transcription after removal of rapamycin from the culture medium. I therefore grew cells from the Δ*not4* strain and from its isogenic wild type strain *(BY4741*) to the appropriate exponential phase of growth. At this point, I added rapamycin to the medium and incubated for 1 hour at 30°C. In order to test the kinetics of transcription reactivation, I removed rapamycin by cell centrifugation and resuspended the cells in fresh medium. Next I sampled cells at different times after rapamycin removal and extracted total RNA for Northern blot analysis. Fig.4, panel A shows the result of Northern blots probed with *RPL30* and *RPL11a* cDNA and panel B displays the quantitation of data, as normalized to ribosomal RNA. As it is clear from the graph in panel B, in the wild type strain RP genes are significantly repressed by rapamycin but ,after removal of the repressor, recover full transcriptional rate in within 4 h. In contrast to the wild type strain, the Δ*not4* strain is not able to efficiently restore transcription. Analysis of cell growth (Fig 4C), is consistent with the transcriptional observation and clearly shows that the wild type strain accumulates a delay of about two hours before returning to a normal growth rate, while cells of the Δ*not4* strain, which grow slower even in the absence of rapamycin, do not show an efficient recovery of growth in

four hours after treatment with rapamycin. In Fig 4D is reported a FACS analysis of the cell cycle, carried out after staining the cells with propidium iodide (PI). PI intercalates in the genomic DNA, and allows to quantify the amount of cellular DNA in order to determine the phase of the cell cycle in which the cells are distibuted. Data from this experiment demonstrate, consistently with previous data, that the Δ*not4* cells accumulate in G1 phase for at least two hours after treatment with rapamycin, while wild type cells are more rapid in proceeding to the S phase of the cell cycle.

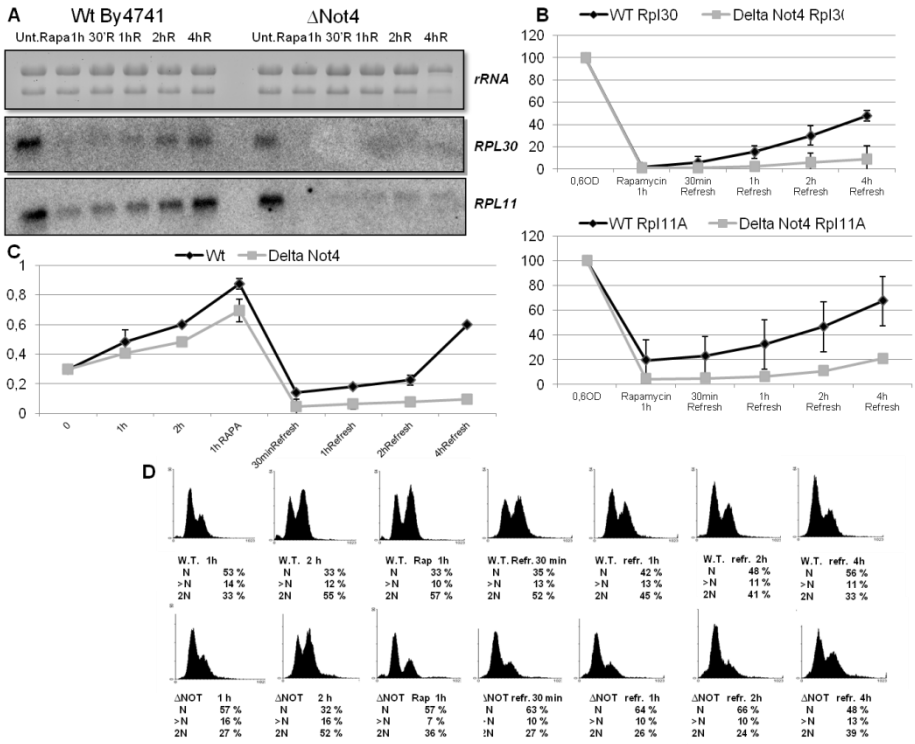


Fig. 4 Panel A: Northern blot showing *RPL30* and *RPL11A* mRNA accumulation in normal growth, at one hour post-rapamycin addition to the medium and at different times from rapamycin removal. Panel B: Quantitation of *RPL30* and *RPL11A* mRNA at one hour post rapamycin addition to the medium and at different times from rapamycin removal. Panel C: Delta *not4* and wt strains growth curves before and after addition and removal of rapamaycin. Panel D: Fax analysis of propidium iodide stained cells from *not4* and wt

strains taken before and during rapamycin treatment or at the indicated times from rapamycin removal.

Proteasome association is strongly decreased in a *not4* deleted strain

My hypothesis which remains to be proven is that Not4 regulates H3K4 methylation through proteolysis of the Jhd2 demethylase. This mechanism requires a coordinated action of Not4 and Ubc4 which leads to Jhd2 ubiquitylation, therefore promoting proteasome recruitment on RP genes chromatin with subsequent Jhd2 degradation.. The proteaosme as described above, is composed of a catalytic 20S proteasome cylinder, which encompasses Pre1 catalytic subunit. Thus, I have analyzed by ChIP the association of Pre1 with the promoter and *RPL24A/RPL30* ORFs chromatin regions in standard growth conditions and after the addition of rapamycin. Fig 5 A and panel B shows that in a wild type strain the chromatin association of Pre1 is detectable in normal conditions on the promoter and ORFs as previously shown (Auld et al. 2006). This association is even more evident after rapamycin repression, especially at the level of the promoter and the 5 'coding regions of *RPL30* (panel A) and *RPS12* (panel B). On the other hand, the binding of Pre1 is much reduced, both during standard growth and after

repression, in the Δ*not4* strain (Fig 5 panel A and B), suggesting requirement of the Not4 protein for an efficient proteasome recruitment on the chromatin regions.

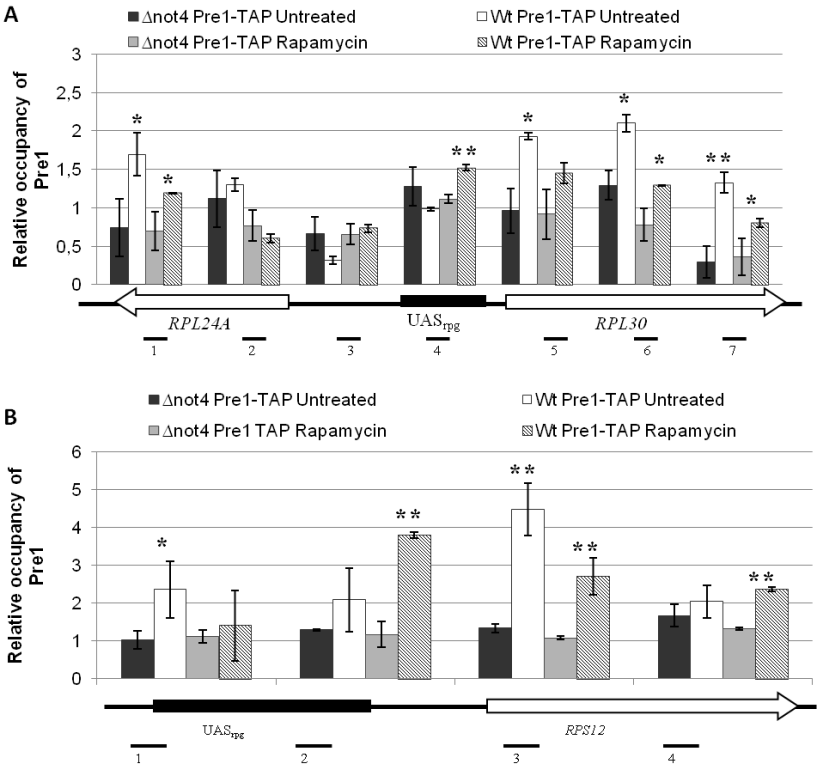


Fig. 5 Panel A: Relative occupancy of Pre1 at *RPL24A/RPL30* transcription unit in wt or delta *not4* strains during normal growth or after the addition of rapamycin. Panel B: Relative occupancy of Pre1 at Rps12 transcription

units in wt or delta *not4* strains during normal growth or after the addition of rapamycin.

Influence of Proteasome inhibition on Tri-Methyl H3K4 level.

In the previous section, I showed that Not4 is required for efficient proteasome recruitment at RP genes. It remains to prove that this recruitment reflects a specific function of the proteasome in the degradation of Jhd2 and consequent regulation of H3K4 methylation.

To test the influence of proteasomal function on the tri-methylH3K4 level we used a yeast strain deleted in the gene coding for the Erg6 protein. This protein is a Delta(24)-sterol C-methyltransferase which converts zymosterol to fecosterol in the ergosterol biosynthetic pathway by methylating position C-24; localized to both lipid particles and mitochondrial outer membrane. The deletion confers an higher permeability to the cell membrane and is useful to introduce in the cells MG-132 a specific proteasome inhibitor in order to observe its effects on H3K4 methylation..

So I grew these cells in YPD standard medium, in addition to rapamicyn for 1 hour, with or without MG-132. Samples were extracted and immunoprecipitated with anti-tri-methyl H3K4

antibodies to determine the H3K4 trimethylation pattern on *RPL24A/RPL30*..

The graph in Fig 6 shows the results. It is clear that following the addition of rapamycin to the culture medium or after separate addition of MG-132, no significant variation of the tri-methyl H3K4 pattern previously observed for the WT strain (see Fig 1) is obtained. On the contrary, 1 hour treatment with both rapamicyn and MG-132 produces a dramatically decrease of H3K4 tri-methylation. This result is consistent with the hypothesis that the proteasome degrades Jhd2 demethylase so that when its catalytic activity is inhibited by MG-132 and transcription is repressed by rapamycin with consequent blockage of Set1 methylation, a consistent H3K4 demethylation is observed..

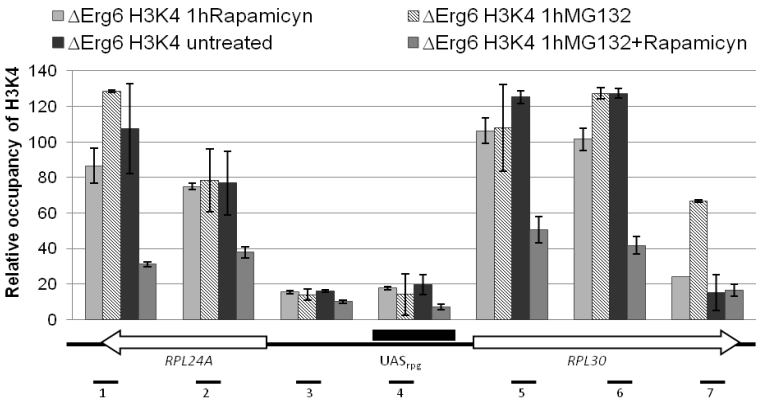


Fig. 6 Proteasome inhibition leads to increased demethylation of *RPL24A/RPL30* in repressed conditions

Not4 and Jhd2 interplay determines H3K4 methylation state at *RPL24A/RPL30* transcription unit.

To better understand the functional role of Not4 and Jhd2 association with *RPL24A/RPL30* transcription unit, we tested the state of H3K4 methylation in other genetic backgrounds:

a. a *Δnot4/Δjhd2* strain
b. *Δnot4/Δjhd2* strain carrying a plasmid with an episomal copy of the *JHD2* gene under the control of its own promoter.

We first tested H3 occupancy in normal growth and after rapamycin repression in the two strains and compared it with that one observed in the wild type and in the *Δnot4* strain (Fig 7a panel A). As it is evident, in all four strains, during normal growth, both promoter and coding regions are H3-depleted, as compared with the control amplicon. This is in agreement with previous observations (Bernstein,B.E. et al., 2004; Lee,C.K. et al., . 2004). Rapamycin repression slightly increases H3 occupancy in the coding regions, as previously observed, but does not lead to a substantial change in the promoter region. Next, we compared the H3K4 tri-methylation pattern of the four strains (Fig.7b). As already shown before (Fig.1) in the wild type strain the peak of tri-methylation is localized in the promoter and proximal portion

of *RPL30* ORF (Fig.7b panel A) and rapamycin repression leads to a general decrease of H3K4 tri-methylation. This decrease is much more evident in the *Δnot4* strain (Fig.7b panel B), leading to a 3-fold reduction of H3K4 tri-methylation in repressed condition, as compared with the wild type strain. This data already reported in Fig.2, suggested that the enhanced Jhd2 activity present on the *Δnot4* strain becomes prevalent in repressed condition when is not balanced by Set1 methylating action. This interpretation is now supported by the observation that the H3K4 tri-methylation pattern of the *Δnot4/Δjhd2* strain (Fig.7b panel C), does not considerably change upon repression. The same strain, when transformed with a plasmid carrying a copy of the *JHD2* gene under the control of its own promoter, shows instead a drastic reduction of H3K4 tri-methylation, both in normal growth and in repressed conditions, even as compared with the wild type strain. This could be explained by an increased expression of the episomal copy of the gene as compared with the chromosomal copy of the wild type strain which indeed I experimentally observed (Fig.7b, panel E). Next, I tested the H3K4 di-methylation levels. The *Δnot4/Δjhd2* strain carrying the episomal copy of *JHD2* shows a drastic reduction of di-methyl H3K4 in both conditions (compare panel 7C with 7D) which suggests that,

when over-expressed, the demethylase is active also on di-methyl H3K4, as previously suggested (Tu,S et al., 2007).

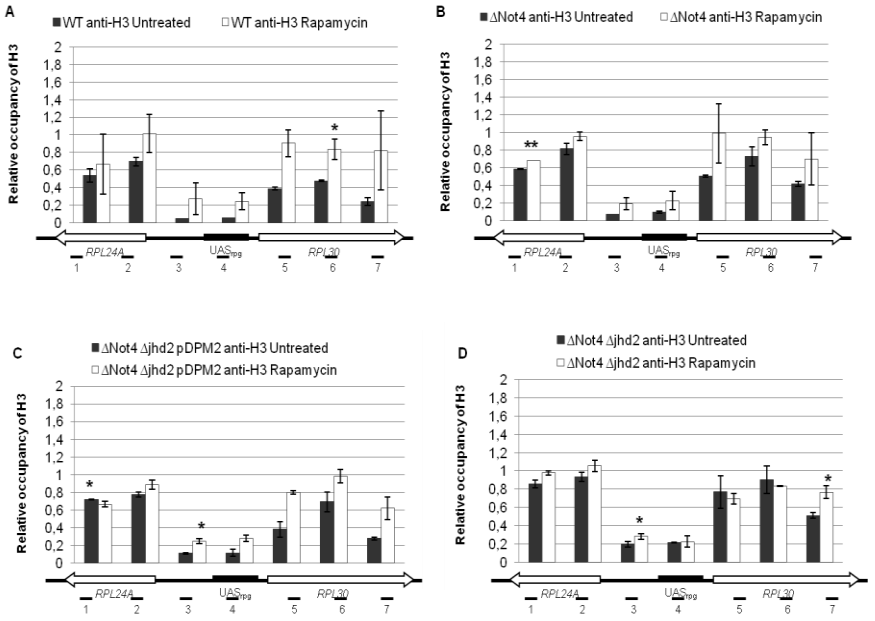


Fig. 7a H3-deplection in all four considered strains.

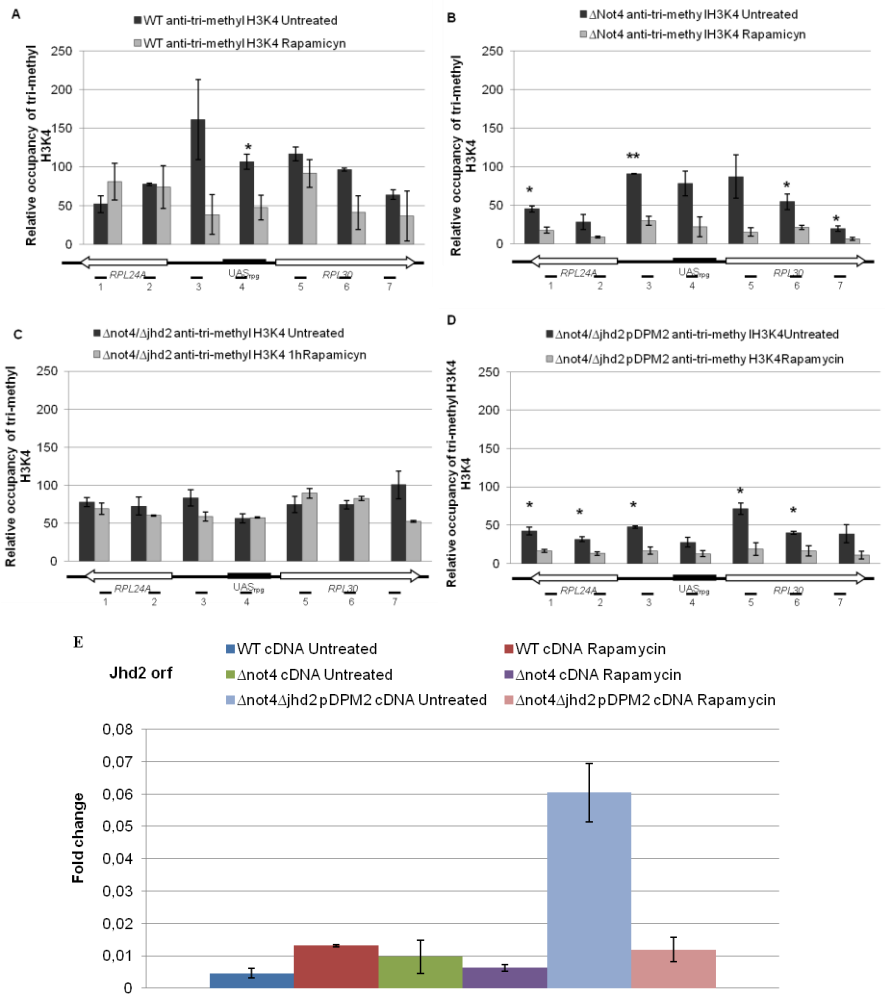


Fig. 7b Panel A. Relative histone H3K4 tri-methylation in the wild type strain during normal growth or in repressed condition. Panel B. Relative histone H3K4 tri-methylation in the *Δnot4* strain during normal growth or in repressed condition. Panel C. Relative histone H3K4 tri-methylation in

the *Δnot4/Δjhd2* strain during normal growth or in repressed condition. Panel D. Relative histone H3K4 tri-methylation in the *Δnot4/Δjhd2* strain transformed with plasmid PDM2 during normal growth or in repressed condition. Panel E. Espression of JHD2 in the different genetic backgrounds. Real time RT-PCR was performed on cDNA obtained from total RNA extracted from the indicated strains during normal growth or 1h post rapamycin addition to the medium.Data represent fold change relative to actin, used as endogenous calibrator. For the untrasformed *Δnot4/Δjhd2* strain no amplification was obtained.

Absence of both not4 and jhd2 proteins seems to cause elongation defects. Study of Rpb1 localizzation and distribution pattern of acetylation.

Not4 was recently shown to have a clear effect in transcriptional elongation (Kruk et al., 2011). I tested our four strains for eventual defects in transcriptional elongation of RP genes. I used RNA polymerase distribution along the ORF, as measured by Rpb1 ChIP, as an assay for elongation efficiency. In this perpective an elongation block determines an increase in the ratio of the Rpb1 localized at the 5' extremity of the ORF, relative to that one localized at the 3' extremity (Gaillard et al., 2009). As shown in Fig.8, the

⊿not4 strain shows indeed an increased ratio between Rpb1 localized in the 5'-extreme amplicon of RPL30 ORF and Rpb1 localize at the 3'-extremity, as compared with the wild type strain. Interestingly, this ratio is even higher in the *⊿not4⁄⊿jhd2* double mutant, while episomal expression of Jhd2 in the same strain restores the wild type situation. A possible interpretation of this experiment is that Jhd2 overexpression is able to suppress the elongation defect caused by the absence of Not4. This could be related to the ability of overexpressed Jhd2 to substantially lower H3K4 di-methylation level.

It has previously been proposed that di-methyl H3K4 is a signal for the recruitment of histone deacetylase at actively transcribed genes coding regions (Morillon,A, et al., 2005; and Mellor J. et al., 2011). Histone deacetylation at coding regions is required to restrict hyper-acetylated chromatin regions to promoters but could make elongation more difficult, especially when Not4 activity is decreased or absent. In figure 9 panel A I show the H3K4 dimethylation level of *Δnot4/⊿jhd2* strain*, as* compared with that one of the same strain carrying the episomal copy of Jhd2. It is evident that the episomal expression of Jhd2 leads to a severe reduction of H3K4 di-methylation. It is possible that this decrease in di-methyl H3K4 in the RP genes coding regions

makes easier for the transformed *Δnot4/Δjhd2* strain to overcome transcription elongation defects deriving from the absence of a functional Not4 by causing an increase of H3 acetylation levels. So I tested the pattern of H3 pan-acetylation in the *RPl30* promoter and ORF for the four different genetic backgrounds in standard growth condition. As seen in fig. 9 panel B, this preliminary data suggest that H3 acetylation of *Δnot4/Δjhd2* strain transformed with plasmid PDM2 is significantly increased at *RPL30* promoter and coding region as compared with *Δnot4* and *Δnot4/Δjhd2* strains.

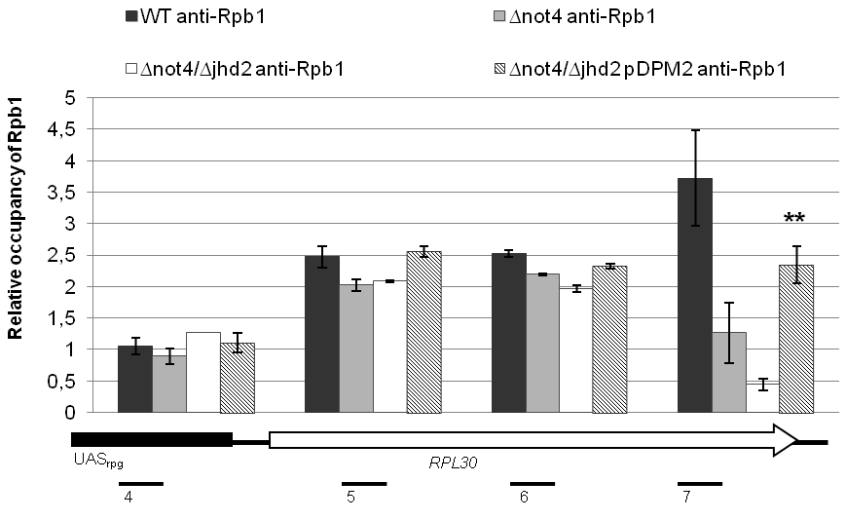


Fig. 8 Relative occupancy of RNA polymerase II at *RPL30* promoter and ORF segments during normal growth.

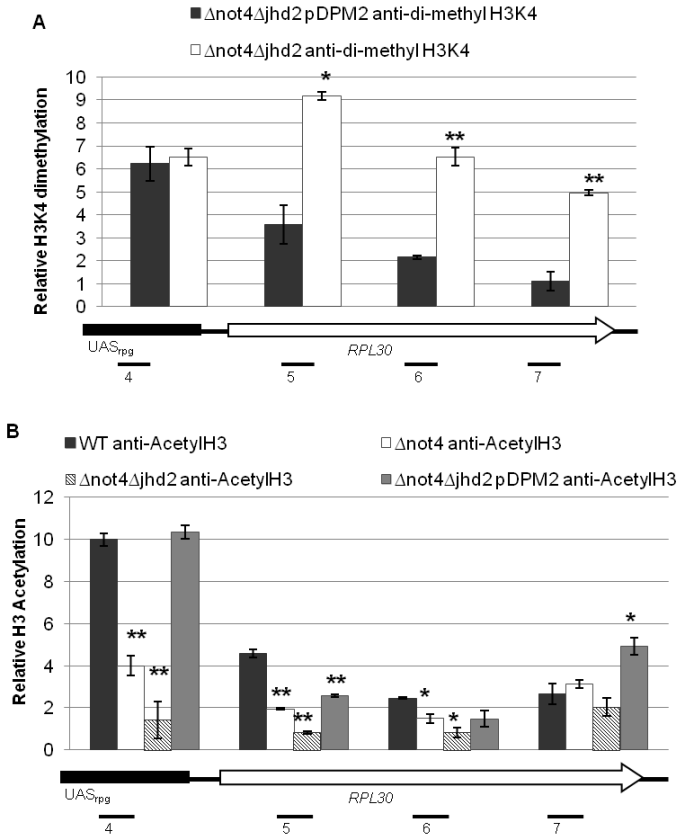


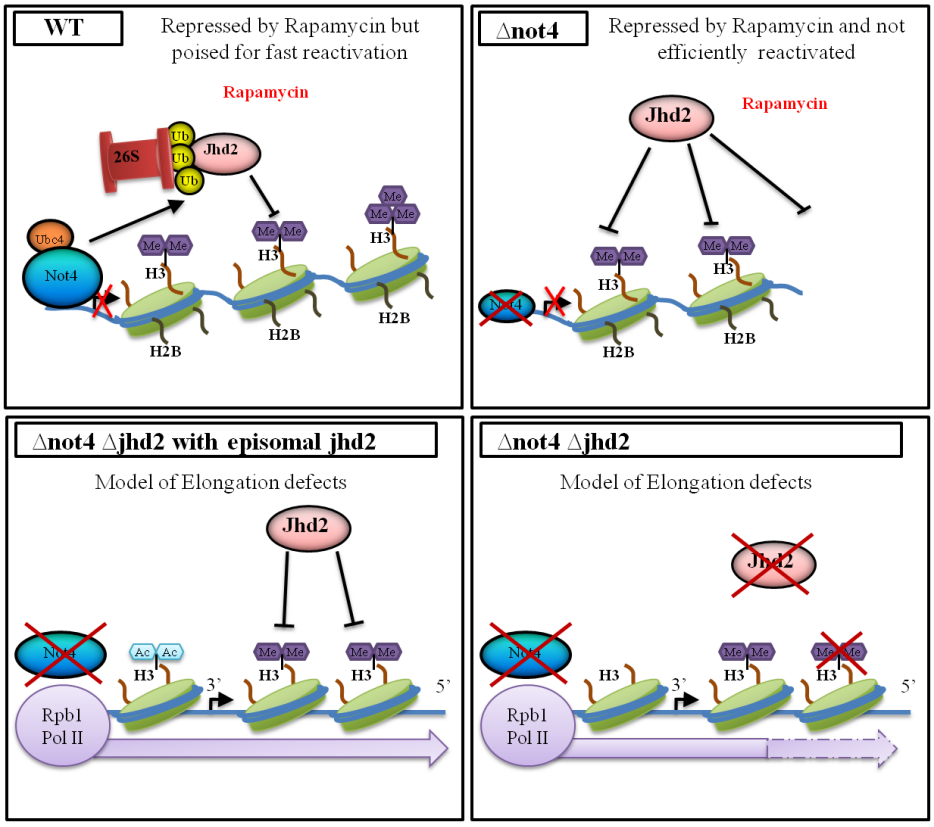
Fig. 9 Panel A Dimethylation level of *Δnot4/Δjhd2* and *Δnot4/Δjhd2* with episomal Jhd2. Panel B. Episomal expression of *JHD2* in the *Δnot4/Δjhd2* strain increase H3 pan-acetylation in *RPL30* promoter and proximal coding region.

DISCUSSION

The chromatin structure of ribosomal protein (RP) genes has peculiar features which are functional to support their high rate of transcription. Among them: high levels of H3K4 tri-methylation and association with 26S proteasome. Not4 was previously involved both in proteasome association and H3K4 methylation (Laribee, et al., 2007; Mersman,P.D et al., 2009). It has been proposed that its action is directly exerted on chromatin sites, promoting Jhd2 demethylase degradation (Mersman,P.D et al., 2009). Jhd2 itself has been found associated with active transcription units (Tu, et al., 2007). I provide unprecedented evidence that both Not4 and Jhd2 associate with the promoter of RP genes, particularly in repressed condition. Jhd2 association increases in a *Δnot4* strain, coherent with a net decrease of H3K4 tri-methylation. On the other end, a *Δnot4/Δjhd2* strain does not show any significant decrease in H3K4 tri-methylation after repression. These observations support a crucial role of Not4 localized at RP genes transcription units in targeting Jhd2 to degradation and consequently in regulating H3K4 tri-methylation. Indeed, a strain treated with the proteasomal inhibitor MG-132 and rapamycin shows a decrease of H3K4 tri-methylation comparable with that

observed in the *Δnot4* strain (Fig. 6). Moreover, association of the proteasome with RP genes transcription units is significantly decreased in the *Δnot4* strain (Fig. 5). This role of Not4 in preventing H3K4 demethylation could be particularly important when RP genes are repressed and the Set1 methylase activity is switched off. In this condition, it is important to maintain a certain level of tri-methyl H3K4 to allow an efficient transcriptional restart as soon as the environment requires it (Fig.10 top panels). This could be one of the reasons which cause a difficult reactivation of transcription and growth in the *Δnot4* strain after rapamycin removal. It may not be the only reason since Not4 is a highly pleiotropic protein which has been involved in many other tasks including transcription elongation. Indeed I found that the *Δnot4* strain has a defect in transcriptional elongation of RP genes. Surpringly, this defect is suppressed by Jhd2 over-expression. How can that happen? My data suggest a role of Jhd2 also in the regulation of di-methyl H3K4 levels. Indeed these levels are significantly decreased in the *Δnot4/Δjhd2* strain when it carries an episomal copy of *JHD2* (Fig.9A). It is possible that this decrease in di-methyl H3K4 in the RP genes coding regions makes easier for the transformed *Δnot4/Δjhd2* strain to overcome transcription

elongation defects deriving from the absence of a functional Not4. It has previously been proposed that di-methyl H3K4 is a signal for the recruitment of histone deacetylase at actively transcribed genes coding regions (Morillon, et al., 2005; Mellor J et al., 2011). Histone deacetylation at coding regions is required to restrict hyper-acetylated chromatin regions to promoters but could make elongation more difficult, especially when Not4 activity is decreased or absent. A preliminary experiment seems to confirm this hypothesis. I analyzed relative H3 pan-acetylation in the promoter and proximal *RPL30* coding region in the four different genetic backgrounds. As shown in Fig.9B, H3 acetylation is significantly increased at promoter and coding region in the *Δnot4/Δjhd2* strain carrying an episomal copy of *JHD2* as compared with all the other strains. This sussudiary effect of Jhd2 in the absence of Not4 is summarized in form of cartoon in Fig.10, bottom panels. Further work will be required to understand the complex interplay between histone modifications which is at the base of the novel and intriguing genetic interaction between Not4 and Jhd2 which we describe here.


WT
Repressed by Rapamycin but poised for fast reactivation
Rapamycin
26S
Ub
Jhd2
Ubc4
Not4
Me
H3
H2B
Δnot4
Repressed by Rapamycin and not efficiently reactivated
Rapamycin
Jhd2
Δnot4 Δjhd2 with episomal jhd2
Model of Elongation defects
Jhd2
Not4
Ac
H3
3'
5'
Rpb1
Pol II
Δnot4 Δjhd2
Model of Elongation defects
Jhd2

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